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CONTENTS

No. 1, SEPTEMBER 20, 1931

	PAGE
THOMPSON, WILLIAM R., JOHNSON, CARL E., and HUSSEY, RAYMOND. A viscosimetric method of estimating enzyme concentration with special reference to amylase.	1
THOMPSON, WILLIAM R., and HUSSEY, RAYMOND. The effect of radiations from a mercury arc in quartz on enzymes. II. The effect of ultraviolet radiation on amylase in solution. . .	9
HOAGLAND, H., and CROZIER, W. J. Geotropic excitation in <i>Helix</i>	15
NORTHROP, JOHN H. The presence of a gelatin-liquefying enzyme in crude pepsin preparations.	29
GOWEN, JOHN W., and TOBEY, ELMER R. Studies on milk secretion. The influence of inanition.	45
GOWEN, JOHN W., and TOBEY, ELMER R. On the mechanism of milk secretion. The influence of insulin and phlorhizin. . .	67
TANG, PEI-SUNG. Temperature characteristics for the production of CO ₂ by germinating seeds of <i>Lupinus albus</i> and <i>Zea mays</i>	87
BARMORE, MARK, and LUCK, JAMES MURRAY. The rôle of phosphate in biological oxidations.	97
SHOUP, CHARLES S., and BOYKIN, JAMES T. The insensitivity of <i>Paramecium</i> to cyanide and effects of iron on respiration. . .	107

No. 2, NOVEMBER 20, 1931

COLE, WILLIAM H., and ALLISON, JAMES B. Stimulation by hydrochloric acid in the catfish, <i>Schilbeodes</i>	119
HITCHCOCK, DAVID I. The combination of a standard gelatin preparation with hydrochloric acid and with sodium hydroxide.	125
HARVEY, E. NEWTON. Photocell analysis of the light of the Cuban elaterid beetle, <i>Pyrophorus</i>	139

CONTENTS

PAGE

HARVEY, E. NEWTON, and LOOMIS, ALFRED L. High speed photomicrography of living cells subjected to supersonic vibrations.....	147
TAUBER, HENRY, and KLEINER, ISRAEL S. Studies on crystalline urease. IV. The "antitryptic" property of crystalline urease.....	155
GREENBERG, David M., and MACKEY, M. A. The sol-gel transformation in gelatin.....	161
WHITAKER, D. M. On the rate of oxygen consumption by fertilized and unfertilized eggs. I. <i>Fucus vesiculosus</i>	167
WHITAKER, D. M. On the rate of oxygen consumption by fertilized and unfertilized eggs. II. <i>Cumingia tellinoides</i>	183
WHITAKER, D. M. On the rate of oxygen consumption by fertilized and unfertilized eggs. III. <i>Nereis limbata</i>	191
CROZIER, W. J., and PINCUS, G. Analysis of the geotropic orientation of young rats. III. Part 1.....	201
CROZIER, W. J., and PINCUS, G. Analysis of the geotropic orientation of young rats. III. Part 2.....	225
CROZIER, W. J., and PINCUS, G. Analysis of the geotropic orientation of young rats. IV.....	243
No. 3, JANUARY 20, 1932	
RAHN, OTTO. A chemical explanation of the variability of the growth rate.....	257
ABRAMSON, HAROLD A. Electrokinetic phenomena. V. A small but constant source of error in measurements of viscosity....	279
LUYET, BASILE J. Variation of the electric resistance of plant tissues for alternating currents of different frequencies during death.....	283
RASHEVSKY, N. On the physical nature of "cytotropism" and allied phenomena and their bearing on the physics of organic form.....	289
GRUNDFEST, HARRY. The sensibility of the sunfish, <i>Lepomis</i> , to monochromatic radiation of low intensities.....	307
NORTHROP, JOHN H., and KRUEGER, ALBERT P. The rôle of intracellular bacteriophage in lysis of susceptible staphylococci.....	329

UPTON, MORGAN. The effect of added loads upon the geotropic orientation of young guinea pigs.	333
ANSON, M. L., and MIRSKY, A. E. The effect of denaturation on the viscosity of protein systems.	341
WYCKOFF, RALPH W. G. The killing of colon bacilli by ultra-violet light.	351

No. 4, MARCH 20, 1932

KRUEGER, A. P. The heat inactivation of antistaphylococcus bacteriophage.	363
HECHT, SELIG, and WOLF, ERNST. Intermittent stimulation by light. I. The validity of Talbot's law for <i>Mya</i>	369
EMERSON, ROBERT, and ARNOLD, WILLIAM. A separation of the reactions in photosynthesis by means of intermittent light. . .	391
CROZIER, W. J., and PINCUS, G. Analysis of the geotropic orientation of young rats. V.	421
CROZIER, W. J., and PINCUS, G. Analysis of the geotropic orientation of young rats. VI.	437
FERGUSON, A. L., and SCHLUCHTER, A. W. The combining weight of gelatin as an acid.	463
FERGUSON, A. L., and SCHLUCHTER, A. W. The mobility of the gelatinate ion.	477

No. 5, MAY 20, 1932

CASTLE, E. S. On "reversal" of phototropism in <i>Phycomyces</i> . . .	487
NELSON, J. M., PALMER, ELIZABETH T., and WILKES, B. G. Similarity of the kinetics of invertase action <i>in vivo</i> and <i>in vitro</i>	491
LINEWEAVER, HANS, BURK, DEAN, and HORNER, C. KENNETH. The temperature characteristic of respiration of <i>Azotobacter</i> . .	497
GRUNDFEST, HARRY. The spectral sensibility of the sunfish as evidence for a double visual system.	507
DAMON, E. B. Dissimilarity of inner and outer protoplasmic surfaces in <i>Valonia</i> . III.	525
JACQUES, A. G., and OSTERHOUT, W. J. V. The accumulation of electrolytes. IV. Internal <i>versus</i> external concentrations of potassium.	537

	PAGE
PALMER, ALBERT H. The adsorption of gelatin by collodion membranes.....	551
TANG, PEI-SUNG. On the respiratory quotient of <i>Lupinus albus</i> as a function of temperature.....	561
TANG, PEI-SUNG. A respirometer vessel for study of metabolism of seeds.....	571
ABRAMSON, HAROLD A. Electrokinetic phenomena. VI. Relationship between electric mobility, charge, and titration of proteins.....	575
ABRAMSON, HAROLD A., and GROSSMAN, E. B. Electrokinetic phenomena. VII. Relationship between electric mobility, charge, titration curve, and optical rotation of protein.....	605

No. 6, JULY 20, 1932

COLE, WILLIAM H. Stimulation by the salts of the normal aliphatic acids in the rock barnacle <i>Balanus balanoides</i>	611
ALLISON, JAMES B. Stimulation by hydrochloric acid and by the normal aliphatic acids in the sunfish <i>Eupomotis</i>	621
STONE, FLORENCE M., and COULTER, CALVIN B. Porphyrine compounds derived from bacteria.....	629
COLE, KENNETH S. Electric phase angle of cell membranes.....	641
HOLLENBERG, G. J. Some physical and chemical properties of the cell sap of <i>Halicystis ovalis</i> (Lyngbye) Areschoug.....	651
TANG, PEI-SUNG. The effects of CO and light on the oxygen consumption and on the production of CO ₂ by germinating seeds of <i>Lupinus albus</i>	655
OSTERHOUT, W. J. V., and STANLEY, W. M. The accumulation of electrolytes. V. Models showing accumulation and a steady state.....	667
MARTIN, DONALD S. The oxygen consumption of <i>Escherichia coli</i> during the lag and logarithmic phases of growth.....	691
BLAIR, H. A. On the intensity-time relations for stimulation by electric currents. I.....	709
BLAIR, H. A. On the intensity-time relations for stimulation by electric currents. II.....	731
INDEX TO VOLUME 15.....	757

A VISCOSIMETRIC METHOD OF ESTIMATING ENZYME CONCENTRATION WITH SPECIAL REFERENCE TO AMYLASE

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(Accepted for publication, June 12, 1931)

A viscosimetric method for the estimation of trypsin concentration was developed by Northrop and Hussey.¹ Since that time it has been employed for the estimation of trypsin and pepsin concentration by Hussey and Thompson,² and Northrop and Clark.³ Certain modifications of the original technique were employed in some of these investigations; but, owing to the fact that interest was focussed primarily upon the effects of radiations upon enzymes, some of these modifications have not been reported in detail.

The present purpose is to outline these modifications and to give the results of investigations whose object was to apply similar technique to the estimation of the concentration of amylase. This method depends upon the action of amylase upon a starch solution. The preliminary observations from which it was developed were made in our laboratory in 1923.⁴ Since that time, other workers⁵ have used a viscosimetric method of this type for the estimation of amylase concentration. However, in their published results the variation admitted in the mixing ratio with consequent variation in starch con-

¹ Northrop, J. H., and Hussey, R. G., *J. Gen. Physiol.*, 1922-23, 5, 353, 355.

² Hussey, R. G., and Thompson, W. R., *J. Gen. Physiol.*, 1922-23, 5, 647; 1923-24, 6, 1; 1925-26, 9, 309, 315, 217.

³ Clark, H., and Northrop, J. H., *J. Gen. Physiol.*, 1925-26, 9, 87.

⁴ Unpublished.

⁵ Davison, W. C., *Bull. Johns Hopkins Hosp.*, 1925, 37, 281. Maslow, H. L., and Davison, W. C., *J. Biol. Chem.*, 1926, 68, 75. O'Donovan, C., and Davison, W. C., *Bull. Johns Hopkins Hosp.*, 1927, 40, 238. Elman, R., and McCaughan, J. M., *Arch. Int. Med.*, 1927, 40, 58.

centration is objectionable, as is also the primary mixing of serum and substrate solution in the viscosimeter itself.

TECHNICAL PROCEDURES

The viscosimeters employed were of the Ostwald type. However, for the proposed work certain deviations from the usual design seemed advantageous, particularly the placement of the start and finish marks upon non-capillary tubing. The object of this was to prevent the formation of air bubbles. Two types of viscosimeters were used, which will be designated as the 5 ml. and the 10 ml. types, respectively, corresponding to the volumes of fluid for which they are designed. Those of the 10 ml. type were made in this laboratory and were adjusted to a 60 second water outflow time at 34°C. These viscosimeters were employed in previous work as well as in that to be reported at present. More recently a 5 ml. viscosimeter, otherwise of the same characteristics, has been employed with satisfactory results.

While measurements were being made, the viscosimeters were immersed in a vertical position so that the start line (upper mark) was between 3 and 4 cm. below the surface in a thermoregulated water bath, the temperature of which was restricted to an interval of 0.06°C. in the usual manner. In the case of the starch-amylase investigation this interval was $37.5^{\circ} \pm .03^{\circ}\text{C}$. A 10 gallon rectangular glass battery jar was used as a container. The water level of the bath was made sensibly constant without interference with thermoregulation by means of a connection with a reservoir *via* an automatic float-operated valve.

Preliminary observations were made to find a suitable temperature, hydrogen ion concentration, substrate concentration, autoclaving technique, and substrate-enzyme mixing procedure. Early in this work it became obvious that serious difficulty is to be encountered in any attempt to fix reproducible standards. Accordingly, as a temporary expedient only one lot of starch was employed in the present experiments (Baker's Soluble Starch, Lot number 13128).

A standard 7 per cent starch substrate solution was employed which was made in the following manner except that it was admissible to substitute any proportional set for the gravimetric and volumetric quantities involved.

7 gm. of Baker's Soluble Starch, Lot number 13128, were weighed out and suspended in 65 ml. of redistilled water in a Pyrex Erlenmeyer flask of approximately three or four times that volume. Then, with constant agitation to prevent overheating of the starch, the suspension was heated just to the boiling point; the top of the flask was covered with a double-layered unbleached muslin cap and the whole transferred to an autoclave wherein the temperature was rapidly raised to $120^{\circ} \pm 2^{\circ}\text{C}$. and so maintained for a period of 20 minutes. Then the solution was removed and cooled to a temperature between 50° and 70°C. and so maintained while 30 ml. of M/15 phosphate buffer (pH 6.7 according to Sørensen) was added, and enough redistilled water to make the total volume 100 ml., and the whole mixed and filtered through a single layer of unbleached dry muslin (previously

prepared by thorough washing with redistilled water), the temperature of all components being maintained between 50° and 70°C. throughout. The filtrate was collected in an Erlenmeyer flask, stoppered with a clean rubber stopper, and cooled, and maintained at $37.5^\circ \pm .03^\circ\text{C}.$ until used or discarded.

In any series of enzyme digestions used in a viscosimetric method of studying their progress it is the custom in this laboratory to follow the procedure of Northrop and Hussey¹ in fixing the ratio, R , of volume of enzyme solution to volume of substrate solution to be mixed. In the case of the starch-amylase digestions of the present report $R = 1/5$. In any case the digestion procedure is as follows.

A definite volume, V , of substrate solution is measured into a suitable flask (of capacity at least $4V$) which is carefully stoppered and maintained in a thermostat such as has been described. At a known instant of time a volume RV of enzyme solution is added and the whole thoroughly mixed. The time on a chronometer to the nearest thousandth of an hour is noted at the beginning and at the end of this transfer and the mean of these, τ_0 , is computed. A proper portion of the mixture is transferred to each viscosimeter to be employed, care being taken to avoid undue temperature change and the formation of air bubbles. An attachment is made to each viscosimeter in such manner that by means of a definitely maintained partial vacuum system and a three-way stop-cock the liquid can be raised at will and then allowed to flow through the capillary tube and the outflow time between marks observed with the aid of a calibrated stop-watch. A sequence of such observations is made and the time in hours to the nearest thousandth is read and recorded which corresponds to the instant of passing the upper mark (start line), in each instance employing the same chronometer as for the estimation of τ_0 . For the i -th observation for a given digestion and a given viscosimeter let τ_i be the time so observed, and let s_i be the time in seconds observed for the outflow. Then with s_i we associate t_i defined by the relation

$$(1) \quad t_i = \tau_i - \tau_0 + \frac{s_i}{7200}$$

Obviously, t_i is the interval of time (in hours) elapsed from the mean time of mixing enzyme and substrate (τ_0) to the mean time of observation. The number-pair (t_i, s_i) will be called a D -point and all such points for a given digestion in a given viscosimeter will be said to form a set, D . These points may be charted on coordinate paper (with s as ordinate and t as abscissa) and a smooth curve drawn. By convention this curve is to begin at the s axis and extend continuously as a decreasing function of t with continuous negative but increasing first derivative. The point of intersection of this curve and the s axis may be designated by $(0, s_0)$.

The original procedure of Northrop and Hussey¹, working with trypsin and pepsin gelatin digestions, involved the graphic estimation of s_0 by extrapolation. Hussey and Thompson² succeeded in developing a system (unpublished but employed in their published work) of estimation of s_0 by a sequence of measurements

in the same viscosimeter of the outflow time of a blank consisting of a mixture prepared in the same way as the digestion mixture with the exception that a conventional solution devoid of enzyme was substituted for the enzyme solution (e.g., 0.85 per cent saline).

The importance of s_0 is obvious in view of the following remarks. A number, ϕ , is taken such that $0 < \phi < 1$, and such that there exists a point of every D -set to be used having its ordinate less than S where

$$(2) \quad S = \phi \cdot s_0,$$

and the smooth curve mentioned above has one and only one point with S as ordinate. Then let T be the abscissa of this point. The point, (T, S) , may be called the *threshold crossing point*; the line, $s = S$, the *threshold*, and T the *threshold intercept*.

Now, let Q be defined by

$$(3) \quad Q = \frac{1}{T}$$

where T is as defined above, an approximation of the time (in hours) required for the standard change in viscosity.

It has been shown that for several enzyme-substrate systems Q is at least a fair approximation of enzyme concentration in arbitrary units so chosen that for unit enzyme concentration $T = 1$. In all preceding work and in that reported in the present communication the arbitrary value, $\phi = 0.8$, has been assigned corresponding to 20 per cent change in viscosity.

By the procedure of estimating s_0 for a given digestion curve just before the digestion is started, one of the greatest difficulties due to the personal element in curve plotting is eliminated. Accordingly, the curve is always drawn through the point, $(0, s_0)$, so estimated. However, this point is not called a D -point, but is associated with the D -set.

To decrease further the differences in curve plotting technique due to differences in individual judgement, the following curve plotting rules may be adopted.

The minimum number of D -points shall be five, exclusive of rejected points if any. Not more than three D -points shall be used which have abscissae exceeding T and none shall be used whose abscissae exceed $1.5T$. If (t_i, s_i) is a point of the D -set and there exists a point on the curve having the same ordinate, s_i , let t_i' be its abscissa and let $d_i = t_i - t_i'$ and otherwise let $d_i = t_i$. Now, let N be the number of D -points (unrejected). Then unless

$$(4) \quad |\sum d_i| \leq 0.002 \cdot N \cdot T$$

(where the summation is over the N points) the curve is rejected as a representation of the points and another drawn, and so on until this condition is satisfied, which is always possible. Now, let λ be defined by

$$(5) \quad \lambda = \frac{\sum |d_i|}{N}.$$

Then, if $\lambda > \theta \cdot T$, where θ is a constant previously fixed for all work of a given type, the whole digestion experience represented by this D -set is discarded. In experiments so far completed the value, $\theta = 0.032$, was used. This was arbitrarily fixed but was taken equal to four times the mean value of λ (in hours) obtained from a long experience of similar curve plotting. As a matter of personal experience not more than once in a hundred instances was discarding so indicated.

A single D -point at a time may be rejected under the following conditions.

Let D_j be one of the D -points (tentatively excluded) and let λ' be defined by

$$(6) \quad \lambda' = \frac{\sum |d_i| - |d_j|}{N - 1},$$

Then, if $N-1 > 5$ and $|d_j| > 4\lambda'$, the point may be rejected. The remaining points are treated then as if the point D_j had never existed. Obviously, the process may be repeated as long as the conditions given above are satisfied.

EXPERIMENTAL RESULTS

The demonstration of the relation between enzyme concentration and T , the time required for a prescribed change to take place, may be called the *critical experiment* in any attempt to evolve a viscosimetric method of the type originally described by Northrop and Hussey.¹ In the case of the present amylase investigation such an experiment was made with amylase solutions bearing a known interrelation by dilutions from a common stock solution.

Four such experiments were performed in each of which three amylase solutions, designated by A , B , and C , respectively, were employed. In each instance the Solution A was a 0.005 per cent pancreatin (Parke Davis) solution in 0.85 per cent saline made by allowing some of the pancreatin powder and the proper amount of saline to lie in contact for $\frac{1}{2}$ hour (the quantities being such that a 0.1 per cent suspension was formed), followed by filtration of the mixture and dilution of a portion of this with saline to twenty times its volume. Solution B was a portion of A diluted to twice its volume with saline, and in the same manner C was formed from B . In each experiment two digestion curves were obtained for each of the solutions used. The values of T so obtained are given in Table I together with the mean of each pair, designated by α , β and γ , respectively.

However, as it is apparent that differences in amylase concentration exist between solutions prepared according to the same procedure at different times, a quantity, J , which depends upon all the observations of a given experiment, is employed as a basis of comparison between these experiments; where J is defined by

$$(7) \quad J = \frac{4\alpha + 2\beta + \gamma}{6}.$$

TABLE I

Soln.		Exp. 1	Exp. 2	Exp. 3	Exp. 4
A	1	0.389	0.429	0.353	0.426
	2	0.390	0.498	0.378	0.426
	α	0.390	0.459	0.366	0.426
B	1	0.981	0.971	1.100	1.030
	2	0.890	0.954	0.954	0.964
	β	0.904	0.963	1.027	0.997
C	1	1.782	1.717	1.589	1.870
	2	1.629	1.736	1.572	1.701
	γ	1.706	1.727	1.581	1.786
J		0.846	0.915	0.850	0.914

TABLE II

	Exp. 1	Exp. 2	Exp. 3	Exp. 4	mean	a.d.	A.D.
α'	0.461	0.501	0.430	0.466	0.467	.020	.010
β'	1.068	1.053	1.205	1.092	1.105	.051	.026
γ'	2.016	1.887	1.860	1.954	1.929	.056	.028

Accordingly, we define α' , β' , and γ' for any given experiment by

$$(8) \quad \alpha' = \frac{\alpha}{J}, \beta' = \frac{\beta}{J}, \text{ and } \gamma' = \frac{\gamma}{J}.$$

The values obtained for these variables in the four separate experiments are given in Table II together with the means, $m(\alpha')$, $m(\beta')$, and $m(\gamma')$.

If a reciprocal relation as in (3) held exactly between amylase concentration and T , and the relative concentrations of A , B , and C were 4:2:1, respectively, then we should find $m(\alpha') = 0.5$, $m(\beta') = 1$, and $m(\gamma') = 2$. The differences between these values and those observed may be compared with the precision measure (A.D.) of each, which may be found in Table II also. Accordingly, wherever differences of such relative magnitude may be neglected, we may employ the method as if the reciprocal relation held exactly.

DISCUSSION

In the past a fairly high precision in the estimation of enzyme concentration in the case of trypsin and pepsin has been attained by resorting to a large number of separate estimations and employing the mean of these. It is a matter of prime importance to restrict evaporation of solutions employed and to maintain uniformity in technique. Enzyme solutions should be protected from undue irradiation (*e.g.*, with sunlight) and stored in a refrigerator or low temperature bath when this is admissible. Changes in observers should be made frequently to detect personal bias particularly in curve plotting. The rules given above are obviously arbitrary. Their object is to reduce the effects of personal differences to a point of negligibility. Obviously, however, there are many other possible systems equally, if not more effective in this respect; but most of these which readily suggest themselves offer much more difficulty in application. Obviously, also, certain of these refinements may be neglected in conformity with tolerances which are relatively large.

SUMMARY

Certain technical modifications of the viscosimetric methods as first employed by Northrop and Hussey have been presented. Most of these have been employed with satisfactory results in the irradiation studies of Hussey and Thompson over a period of several years. These are in turn applied to a method of estimation of amylase concentration.

THE EFFECT OF RADIATIONS FROM A MERCURY ARC IN QUARTZ ON ENZYMES

II. THE EFFECT OF ULTRA-VIOLET RADIATION ON AMYLASE IN SOLUTION

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(Accepted for publication, June 12, 1931)

In an earlier report¹ we have given the results of experiments which are concerned with the effects of irradiation of solutions of pepsin with ultra-violet light, wherein these results were compared with similar effects of irradiation with radiations from radon and its radioactive products in dynamic equilibrium with it, wherein also were included studies with other enzymes, namely, trypsin and invertase. Under fixed conditions of irradiation, it was shown that inactivation of the enzyme took place in each instance studied and that the relation between the enzyme concentration, Q , after irradiation and that before irradiation, Q_0 , could be approximated closely in all cases by the relation,

$$(1) \quad Q = Q_0 \cdot e^{-k \cdot W}$$

where W is a variable proportional to the radiant energy liberated by the source of radiation during the irradiation interval, and k is a positive constant (dependent in each case upon the enzyme system used and upon the conditions of irradiation aside from those which determine the power of the source and period of irradiation). Where the power of the source is constant (or approximately so) then the time, t , of irradiation may be substituted for W in (1) in the general sense there employed, though it should be borne in mind that if a fixed energy unit system for W in a given case has been adopted, as, for example, in the case of the β -ray experiments previously reported,²

¹ Hussey, R., and Thompson, W. R., *J. Gen. Physiol.*, 1925-26, 9, 217.

² Hussey, R., and Thompson, W. R., *J. Gen. Physiol.*, 1922-23, 6, 7.

then a change to a proportional variable in place of W should be accompanied by a change in the value of k in inverse proportion. In the case of ultra-violet irradiation of pepsin, wherein the power of the source (a mercury arc in quartz) might be assumed, if not constant, at least to fluctuate so that t is approximately proportional to the energy liberated under the existing conditions of irradiation, we have shown a satisfactory fit of the results obtained to the relation

$$(2) \quad Q = Q_0 \cdot e^{-k \cdot t};$$

or, in differential form,

$$(3) \quad \frac{dQ}{dt} = -k \cdot Q \quad \text{or} \quad \frac{d \log Q}{dt} = -k,$$

which obviously implies a linear relation between the logarithm of the enzyme concentration and the duration of irradiation under such conditions; or, in general, with the variable W .

Recently, we have been concerned in this laboratory with the estimation of active amylase concentration by means of a viscosimetric method described in another communication³ a modification of which is suggested in another report⁴ from this laboratory by Wies and McGarvey. By means of this modified method we have studied the effects of radiations from a mercury arc in quartz upon amylase solutions.

EXPERIMENTAL PROCEDURES

The solutions were prepared from pancreatin in 0.85 per cent saline as previously described,^{3,4} and the irradiation system was essentially the same as that previously employed in the experiments¹ with pepsin mentioned above. Enzyme was irradiated in the same flat bottomed cylindrical quartz tube (about 25 mm. inside diameter, 1 mm. in thickness, and 36 mm. long) placed vertically above a quartz window (approximately 3 mm. thick and 25 mm. in diameter) in the bottom of a thermoregulated water bath at $10.0 \pm .15^\circ\text{C}$., the water of which was freshly distilled (being replaced at least once every 3 days). The same mechanical stirring device was employed to agitate the enzyme solution during irradiation for which the same mercury arc was employed, tilted at a fixed angle

³ Thompson, W. R., Johnson, C. E., and Hussey, R., *J. Gen. Physiol.*, 1931-32, 15, 1.

⁴ Wies, C. H., and McGarvey, S. M., unpublished.

of 30° to the horizontal, and in a position about 19.0 cm. vertically beneath the quartz window of the bath. The amount of enzyme solution irradiated in the present experiments was 5 ml. A control portion of the same enzyme solution was kept in the same bath in a light-screened container.

The results of a number of such irradiations are given in Table I. Successive estimations upon the control solution showed that the rate of spontaneous inactivation was negligible with respect to the rate of the radiochemical change. Accordingly, Q_0 is taken in each instance as the concentration of amylase in the control solution at the end of the irradiation interval. Precise estimates of the rate of spontaneous inactivation of amylase under the control conditions are not available, but it is estimated as about 10 per cent per day; and this is obviously

TABLE I

t (min)	Q_0	Q	$\frac{Q}{Q_0}$	k' (min.) ⁻¹	$k' - k$
1.03	10.45	8.60	0.823	0.189	-0.049
2.00	10.85	6.52	0.601	0.255	+0.017
4.00	9.66	3.85	0.399	0.230	-0.008
6.00	12.64	3.14	0.248	0.232	-0.006
9.00	12.11	1.38	0.114	0.241	+0.003

Taking the approximation, $k = 0.238 \text{ min.}^{-1}$

negligible in the present experiments with respect to a radiochemical change of about 50 per cent in 3 minutes as observed (approximately 3000 times as great). In Table I will be found the corresponding values of t , Q_0 , Q , and $\frac{Q}{Q_0}$ for each irradiation, together with k' —defined as the value of k calculated in each such instance from the formula of (2). The value of k obtained by fitting the curve given by

$$(4) \quad \log \frac{Q}{Q_0} - k \cdot t = 0$$

to the observed points, $(\log \frac{Q}{Q_0}, t)$, by the method of least squares was found to be 0.2376 min.^{-1} . The differences between 0.238 and the observed values of k' are given in the same table, where it may be

seen that they decrease in *absolute* value with increase in t , as might be expected.

It may be noted, furthermore, that inactivation has been extended as far as 88 per cent change, approximately. In order to estimate Q in such cases of great change, a flexibility of the viscosimetric method previously described was utilized by replacement of the usual addition of 5 ml. of enzyme to 25 ml. of substrate solution (3 per cent starch substrate) by the addition instead of first x ml. of 0.85 per cent saline and then y ml. of enzyme solution (where $x + y = 5$) to the above amount of substrate. Q is calculated from the resulting value of T (the time in hours for 15.8 per cent change in viscosity as described⁴) for the given digestion curves by the formula

$$(5) \quad Q = \frac{5}{y \cdot T}.$$

DISCUSSION

In the earlier work¹ upon the effects of ultra-violet radiation upon pepsin in solution it was observed in two successive experiences that, although the relation (2) held, it was necessary to introduce different constants for k in each instance. This was supposed to be due to a decrease in the intensity of radiation incident to the irradiated solution. Care was taken in the present experiments as to elimination of and prevention of accumulation of impurities in the water which might induce such decrease in intensity of radiation. The consistent results obtained indicate that the required condition of sensibly constant ratio between the time of irradiation, t , and the energy increment was realized. However, in subsequent work temporary deviations were noted which may be due to variation in the potential difference of the lamp electrodes. Further work in this connection is in progress.

Direct comparison of sensitivity of pepsin and amylase solutions is made impossible in these results due to the lack of definite information as to radiation intensities, but it seems evident that amylase solutions are much more sensitive than are pepsin solutions, perhaps more than 50 times as sensitive.

Further work involving different aspects of the radiochemical

inactivation of amylase is in progress in this laboratory, one of the immediate results of which is a demonstration that sensibly complete protection (within the limits of tolerance of the present work) is given by interposition of a No. 1 Crookes Glass filter (1.7 mm. thick) between the quartz window and the enzyme solution.

SUMMARY

Amylase in solution is inactivated by the radiations from a mercury arc in quartz, in a manner similar to that previously reported for pepsin. The reaction was followed to a point where more than 88 per cent change had taken place, the course being that of monomolecular radiochemical change. Apparently, this reaction is due to the influence of ultra-violet radiation alone.

GEOTROPIC EXCITATION IN HELIX

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The geotropic orientation of a creeping gasteropod such as *Helix* presents for examination a number of questions which have not always been kept distinct in discussions of geotropism (*cf.* Crozier and Pincus, 1926-27). We are concerned to learn something about geotropism as a function of the circumstances which may govern its expression. It is necessary, therefore, to treat separately (1) the latent period for geotropic response, (2) the speed of orientation, (3) the position of stable progression, (4) the speed of oriented progression, each as a function of the exciting component of gravity. For certain forms the latter is easily varied by altering the slope of the surface upon which creeping takes place. An understanding of the orientation which shall be complete enough to permit utilizing it for the formulation of situations in which, for example, both light and gravity are simultaneous variables, necessitates beyond the investigation of the points just enumerated an understanding of the relations between the innervation and central nervous control of the parietal musculature and of the musculature of the foot as concerned in progression (*cf.* Crozier and Federighi, 1924-25). The present paper is one of several devoted to furthering this analysis. A review of the general situation is given in a paper by Crozier and Navez (1929).

The question here treated has to do with the mechanism of excitation. Such gasteropods as *Helix* are provided with a "statocyst." The operative removal of this organ is difficult, at least without introducing the possibility of other changes. So that to perform an experiment of the sort used by Parker (1927) to test the significance of the sphaeridia of sea-plates for their "right side up" orientation in space, is mechanically impossible. It was found by Wolf (1926-27) that a definite and comprehensible relation subsists between the extent of

upward (or downward) orientation upon an inclined surface and the slope of the surface, in *Agriolimax*, and that the relationship is in principle similar to that apparent in the case of young rodents (Crozier and Pincus, 1926-27; 1929; 1929-30). The successful formulation was based upon the idea that upward orientation ceases, or is no longer forced, when the downward pull of the body mass is so adjusted that within a threshold difference the pull is the same on the two sides. Similar observations have been made subsequently with other gasteropods. This treatment is quite independent of any assumption that the statocyst is the organ whose excitation governs geotropic excitation, but in itself (Crozier, 1928) it says nothing about the possible involvement of the statocyst in some other feature of geotropic excitation or of the response itself.

Experiments by Crozier and Navez (1929) made it clear that the latent period of the upward orientation of *Liguus* and of certain other gasteropods, creeping upon a *vertical* plate, is definitely a function of the pull of the mass carried by the parietal muscles, and that the direction of this pull (*cf.* also Cole, 1925-26) determines the direction of orientation (unless "reversal" takes place). This rules out any quantitative connection between statocyst and geotropic excitation. It does not necessarily signify, of course, that the statocyst is completely devoid of significance for the geotropism. We have more recently undertaken a careful examination of the latent period for the orientation-response as related to the slope of the supporting surface. The results confirm in an adequate manner the correctness of the assumptions underlying the treatment of geotropism as a result of impressed muscle tensions. The experiments are of interest in another way, namely because of their simplicity, while nevertheless possibly giving a fairly direct approach to the physiology of tension-receptors through the investigation of intact organisms.

II

A large plate of ground glass was pivoted at its center so as to be rotatable upon a smooth bearing. The bearing was mounted upon a steel rod so hinged at its base as to be inclinable at any desired angle. On the freshly moistened surface of this plate a snail was allowed to creep as nearly straight upward as possible. During the active creeping the plate was rotated in its own plane in such a way that the axis of the animal was turned to a horizontal position. After a

definite latent interval, the anterior end of the snail then begins to orient upward. When such tests are made in an ordinary laboratory room, or even in a dark-room with sufficient light to permit observation, it is impossible to exclude the effects of photic excitation unless the eyes are removed. Amputation of the eye-stalks suppresses phototropic orientations (*cf.* Wheeler, 1923) and at the same time largely does away with the possibility of anemotropic excitation due to air currents brought into play by the rotation; this is tested by rotations with the plane horizontal. Certain of these points have been discussed in other papers (Crozier and Navez, 1929; Crozier and Cole, 1929-30). (Experiments have also been made with a rotatable plate serving as the bottom of a box completely enclosed by a glass or cellophane top, to eliminate air currents.)

The latent period for the initiation of geotropic response is sufficiently long to permit easy measurement with a stop-watch. The latent period or reaction time is estimated from the moment of half rotation of the plate to its new position, which (as measured independently) occupied in the average 0.35 seconds, until the first appearance of upward turning of the head. With each of the tested individuals this reaction time was measured in twenty trials at each of a series of slopes. It is quickly found that the magnitude of the reaction time depends in a definite and characteristic way upon the slope (α) of the glass plate. It has been pointed out that for the estimation of the statistical significance of mean values of such reaction times it is necessary to work with a definite number of individuals at each value of the independently variable condition, and at each such value to secure a constant number of readings with each individual (*cf.* Crozier and Pincus, 1929-30; Crozier, 1929). Evidences which may be obtained as to the "internal consistency" of such observations are more significant than would be the result of unsystematic attempts to increase formal precision by merely increasing numbers of observations. A chief point in view in our inquiry has been the possibility of determining whether the variability of latent period may not be controlled by the gravitationally exciting vector in the same manner as the latent period itself.

III

Four animals (*Helix lactea*) were used for the most complete series of experiments by the method previously outlined. A variety of tests showed that it was possible to obtain very closely agreeing mean values of the reaction time in series of tests on different days with the same individual. In a number of cases the curves of reaction times were very closely duplicated in such parallel series. The temperature of the laboratory was $20^{\circ} \pm 1^{\circ}$. The four animals chosen each weighed approximately 6 gm.

The body of such a snail as *Helix* of course exhibits a fundamental torsion. We therefore compared reaction times exhibited when the

animal is turned to the *right* and to the *left*. No *consistent* differences were found, and in each case the difference was quite small (*cf.* Crozier and Navez, 1929). The practice was to alternate turnings to right and to left. The successive slopes (α) chosen in a given series of tests were in random order as to magnitude. With adequate periods of rest between trials, of the order of several minutes or more, no evidence could be obtained of any facilitation effects. With lesser intervals facilitation is evident, but other facts point to its origin in the greater extension of the parietal muscles.

TABLE I

Mean Latent Periods for Geotropic Response of Helix lactea as a Function of the Inclination of the Surface (α)

α	R.T.	P.E.
<i>degrees</i>	<i>sec.</i>	<i>sec.</i>
5.0	12.15	± 0.84
7.5	11.15	0.93
10.0	7.12	0.54
15.0	6.22	0.37
20.0	5.03	0.27
30.0	4.95	0.28
34.0	4.90	0.17
40.0	4.13	0.22
50.0	4.30	0.32
60.0	3.91	0.23
70.0	3.41	0.17
80.0	3.21	0.19
83.0	2.90	0.084
85.0	2.84	0.10
87.0	2.90	0.12
90.0	2.88	0.11

Table I contains a summary of the measurements. In each case the latent period for geotropic response shortens with increase of the slope. In at least a rough way, the relationship is hyperbolic between *latent period* and the *sine* of the slope, and can be so fitted (Figs. 1, 3), indicating that the active component of gravity directly determines the velocity of the onset of response. The mean values of the latent periods are plotted in Fig. 1. The degree of scatter of the individual measurements at any one value of the slope is rather small, and the

values for the several individuals tend to agree well among themselves. We have therefore combined the measurements from all. In each case, moreover, it is easily seen that the standard deviation of the mean latent period declines in about the same way as the reaction time it-

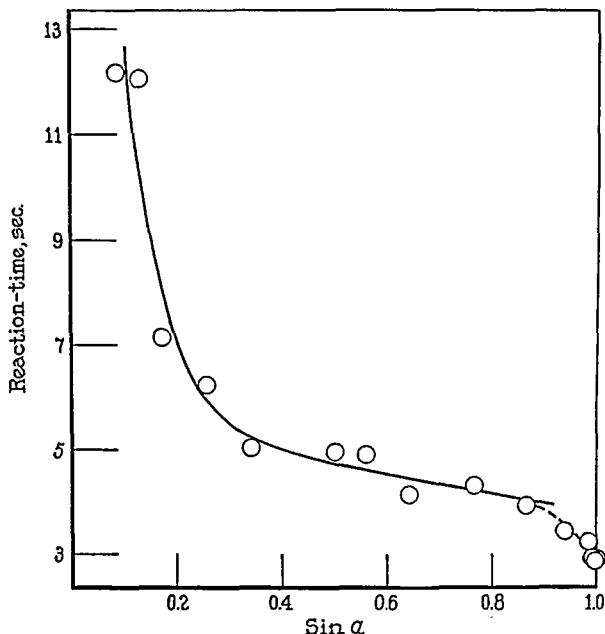


FIG. 1. Mean values of latent period for geotropic response in four individuals (*Helix lactea*), as a function of the slope of the supporting surface (α); twenty observations with each individual averaged for each point (data in Table I).

self declines. With the very highest slopes (above 80°) it is apparent that the reaction time shortens more rapidly with increase of slope than at lower slopes; there is apparent a rather abrupt discontinuity in the curve. Fig. 2 demonstrates that the standard deviations of

the measurements follow a similar course.¹ In fact, both for the collective data and in each individual case the standard deviation of the reaction time is directly proportional to the reaction time itself.

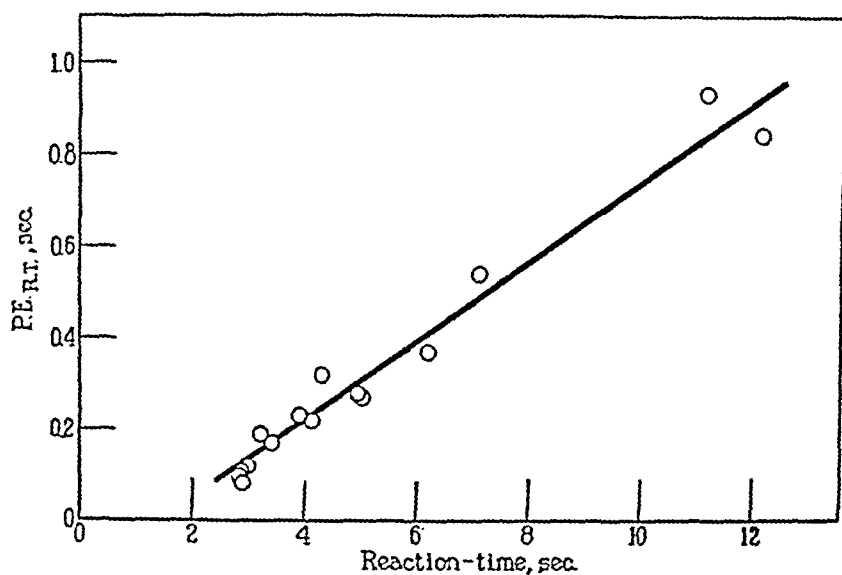


FIG. 2. Probable errors of mean reaction times for geotropic response as a function of the slope of the surface, as related to latent period, with numbers of observations constant (80) at each slope (data in Table I).

TABLE II

A Repetition of the Experiment Summarized in Table I

α	R.T.	P.E.
degrees	sec.	sec.
5	11.12	± 0.67
10	8.48	0.54
15	6.72	0.35
20	5.65	0.27
35	4.51	0.17
50	3.77	0.14
65	2.74	0.11
80	2.08	0.05
90	1.84	0.05

¹ The frequency distribution of measured latent periods at given α is always slightly skewed in the direction of excess of somewhat longer reaction times. We have not found it necessary, however, to allow for this in computing σ . The fact itself is consistent in an interesting way with the idea that what is really measured, and the thing which varies "randomly," is the velocity of the underlying latent-period process, i.e., $1/(\text{Reaction time} - \text{constant})$.

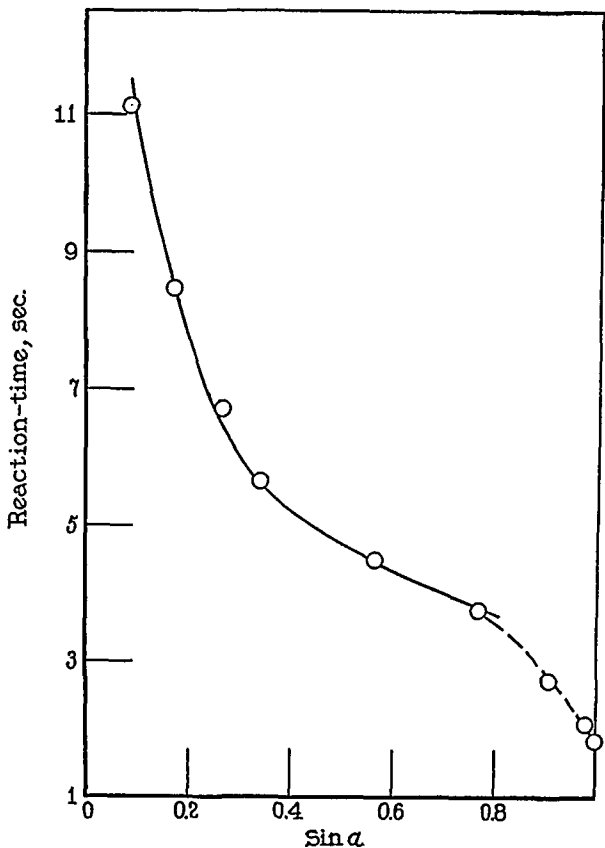


FIG. 3. Mean magnitudes of latent period for geotropic response in four individuals (*Helix lactea*), as in Fig. 1 (data in Table II).

An independent series of measurements, again with 80 observations (twenty on each of four additional individuals) at each slope of surface showed precisely the same relationships (Table II and Fig. 3). We are indebted to Mr. Joseph Berkowitz for assistance in these trials. The probable errors of the mean latent periods are so nearly identical in the two series as to suggest from this additional standpoint the "intrinsic" character of the variation recorded. The connection between latent period and effective gravitational component is again hyperbolic up to about $\alpha = 55^\circ$, and for slopes above this declines sharply. The standard deviation of the reaction time is again directly

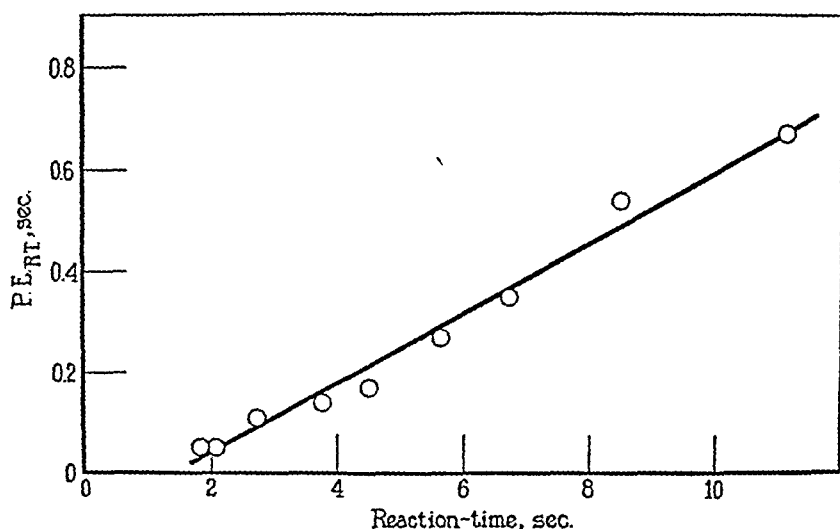


FIG. 4. Probable errors of mean reaction times for geotropic response, as related to the mean magnitudes of the latent periods (data in Table II).

proportional to the reaction time (Fig. 4), so that the *relative variation* of performance [*i.e.*, $\Delta \text{P.E.R.T.} / \Delta (\text{reaction-time})$] is constant.

The alternative mode of procedure in such experiments is to allow the snail to creep on a horizontal plane so pivoted that the plate may be tilted on an axis parallel to the long axis of the snail. Extensive tests of this sort have been made with *Helix nemoralis*. The outcome is in all respects similar to that already described. This technique is capable of certain developments which will be considered in another place.

It is to be noted that whereas series of measurements such as those

here reproduced are typical, one does find that under certain conditions, apparently nutritive, the musculature of the creeping snail may be in such a condition of tonus that the hyperbolic portion of the graph shown in Fig. 2 may descend much more abruptly. With such individuals it may appear that the reaction time of orientation resulting from displacement of the animal's axis at slopes between 40° and 80° may show no significant change, clearly due to the fact that the "minimum latent period" apparent under the conditions is reached at relatively small magnitudes of the gravitationally effective force.

IV

An immediate corollary of the notion that geotropic excitation of gasteropods is due to impressed muscle tensions is, that by modifying these tensions, properties of the geotropic response should show predictable distortion. Several lines of evidence have demonstrated that for *Liguus* (Crozier and Navez, 1929) this is as true as it is for young rats (Crozier and Pincus, 1926-27; 1929-30). Particularly, when the gravitational pull is increased, by attaching masses to the shell, the latent period for the initiation of reaction is decreased. It is not to be lost sight of, however, that the degree of extension of the body muscles (probably both parietal and columellar) modifies the induction of excitation and the speed of response. For example, if repetitions of geotropic excitation by the method of rotating the substratum be carried on in rapid succession (say at intervals of a minute or so), the latent period progressively shortens, then later increases. This is clearly correlated with the progressive extension of the anterior portion of the body leading finally to its becoming flaccid and atonic. In *Liguus* this is not at all so pronounced as in *Helix*, but the response in this case is complicated by the reversal of geotropism induced by the superposition of relatively large loads (Crozier and Navez, 1929).

With *Helix*, buckshot were attached to the shell with bits of adhesive tape, approximately over the center of rotation of the shell in the turning operations. The resulting effects upon the latent periods for geotropic response at various inclinations of the surface may be illustrated by means of data from experiments with one individual (Table III). In evaluating such results it is not to be lost sight of that since the measurements cannot all be made on the same day,

TABLE III
Mean Latent Periods for Geotropic Response of Helix lactea (One Individual, No. 2.1), at Various Inclinations of Support, and with Various Loads Added; Twenty Observations for Each Mean

Added mass		Mean reaction times, sec.								
		$\alpha = 5^\circ$	10°	15°	20°	35°	50°	65°	80°	90°
gm.	0	12.1 ± 0.75	11.9 ± 0.83	7.65 ± 0.25	6.86 ± 0.38	4.90 ± 0.15	4.24 ± 0.12	2.73 ± 0.07	2.07 ± 0.04	1.76 ± 0.04
	1.2	10.8 ± 0.79	7.0 ± 0.54	7.16 ± 0.56	5.58 ± 0.29	3.60 ± 0.15	3.01 ± 0.09	2.70 ± 0.11	2.45 ± 0.08	2.50 ± 0.12
	1.7	12.5 ± 1.19	10.0 ± 0.92	7.02 ± 0.48	4.80 ± 0.27	3.20 ± 0.13	2.67 ± 0.13	1.87 ± 0.05	1.95 ± 0.09	1.85 ± 0.10
	2.2	12.1 ± 0.83	10.2 ± 1.09	7.60 ± 0.62	5.50 ± 0.53	3.78 ± 0.17	2.70 ± 0.10	2.90 ± 0.27	2.06 ± 0.08	2.17 ± 0.11
	2.7	13.3 ± 1.12	10.9 ± 0.97	4.90 ± 0.34	4.70 ± 0.35	4.90 ± 0.42	3.40 ± 0.18	2.20 ± 0.14	2.20 ± 0.11	1.99 ± 0.09

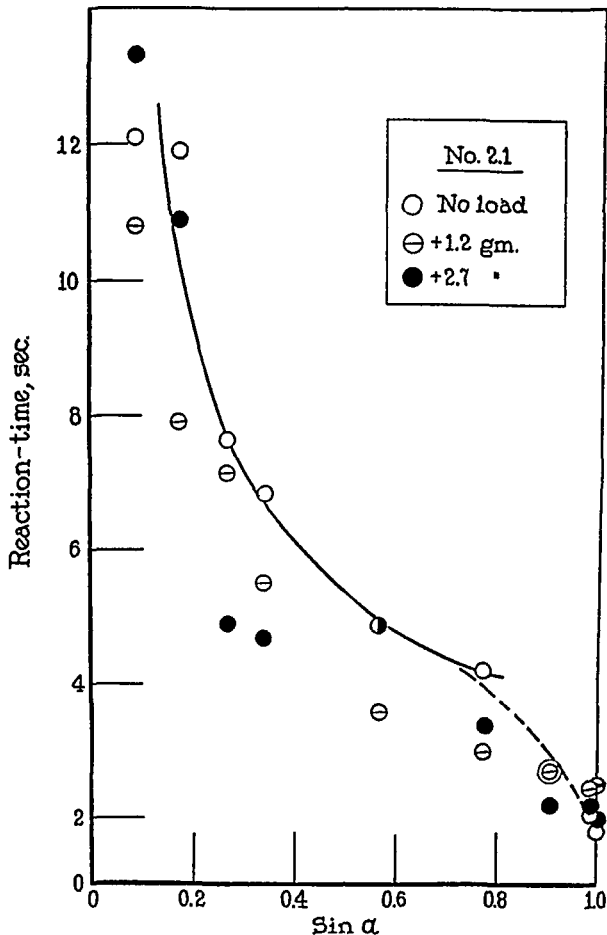


FIG. 5. The relationship of reaction time to inclination of the surface, when the snail bears an additional load (cf. Table III). Records for individual number 2.1 are plotted, without load (curve), and with loads of 1.2 and 2.7 gm.; twenty observations averaged at each plotted point; see text.

fluctuations of responsiveness undoubtedly intervene, although effort is made to keep the snail in comparable condition throughout and to vary the sequences of inclinations and of weights attached. We are under obligation to Mr. Joseph Berkowitz for painstaking assistance in these tests.

We expect the decrease of latent period to be apparent only in the intermediate zone of slopes and of attached masses. This is clearly evident in Table III, and in Fig. 5. The general form of the relationship between latent period and $\sin \alpha$ is identical with added loads and without. Beyond $\alpha = 50^\circ$ the differences are so slight, with the small loads it is mechanically permissible to employ in the present case, as to be without much significance. At $\alpha = 10^\circ$ and $\alpha = 5^\circ$ the effect of the small added masses is not appreciable in this individual, although with others studied in the same way the decrease is more definite. Between $\alpha = 15^\circ$ and $\alpha = 50^\circ$, however, the expected decrease in latent period is real and significant. The elongation of the supporting musculature by the additional load, at high slopes, introduces complications, and is to be expected to lead in this way to a secondary increase of the latent period. The proportionality of σ for latent period to latent period itself is not disturbed by the presence of an additional load (cf. Table III).

It is notable that with the addition of loads to *Helix* the form of the curve connecting *latent period* with $\sin \alpha$ does not change. This is especially striking in the position of the abrupt change of curvature in the region $\alpha = 55^\circ$, which is unaltered. In explanation of this change of curvature (Figs. 1, 3) we have considered that when the slope of the surface exceeds a certain magnitude (about 60° or a little less) the projection of the snail's center of gravity falls outside the base of support when the axis is made horizontal; the animal is then no longer in stable equilibrium, and the forces to be overcome are different in the sense that the adhesion of the foot is called upon to maintain attachment. This condition should not be materially altered with the added loads. The constancy of the location of the "break" in the curve, signifying a much more rapid decrease of latent period with increasing slope of surface, is evidence that the change does not depend upon a critical value of the total intensity of excitation, but rather upon a change in the incidence of stimulation due to the geometry of the situation such as we have supposed.

V

The instances in which it has been possible to demonstrate that variability of response or performance is modified in a manner similar to that in which the magnitude of the response itself is affected by some controlling variable are not as yet numerous. In cases previously analyzed (Crozier and Pincus, *loc. cit.*), from the nature of the response it was necessary to consider the *relative* variation of the measured performance; in the present case it is clearly required to deal with the absolute magnitudes of the standard deviations of the measurements. The conclusion which must be drawn is, that the magnitude of the gravitational excitation determines both the speed of initiation of response and the variability of this speed. When a discontinuity or abrupt change appears in the curve connecting two such variables as exciting force and response, moreover, as in this case, it is a matter of some importance to be able to confirm the reality of this discontinuity through the parallel behavior of the variation of response (*cf.* Crozier, 1929; Upton, 1929-30; Navez, 1930; Pincus, 1930-31).

VI

SUMMARY

Rotation of an inclined surface on which *Helix* is creeping straight upward, such that the axis of the animal is turned at a right angle to its previous position, but in the same plane, leads to negatively geotropic orientation after a measurable latent period or reaction time. The duration of the latent period is a function of the slope of the surface. The magnitude of the standard deviation of the mean latent period is directly proportional to the mean latent period itself, so that the *relative variability* of response is constant. The dependence of reaction time upon extent of displacement from symmetrical orientation in the gravitational field is found also by tilting the supporting surface, without rotation in the animal's own plane. On slopes up to 55°, the relation between latent period and the sine of the slope is hyperbolic; above this inclination, the latent period sharply declines. This change in the curve is not affected by the attachment of moderate loads to the snail's shell (up to 1/3 of its own mass), and is probably

a consequence of loss of passive stable equilibrium when rotated. When added loads do not too greatly extend the snail's anterior musculature, the latent period for the geotropic reaction is decreased, and, proportionately, its σ . These facts are discussed from the standpoint that geotropic excitation in these gasteropods is due to impressed muscle-tensions.

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THE PRESENCE OF A GELATIN-LIQUEFYING ENZYME IN CRUDE PEPSIN PREPARATIONS

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INTRODUCTION

In the course of a study of the fractionation of crude pepsin preparations it was found that amorphous precipitates could be obtained which were extremely active with respect to liquefaction of gelatin (1). In the study of the properties of the crystalline protein isolated from crude pepsin preparations it was also noted occasionally that a small amount of amorphous material was obtained which had this strong gelatin-liquefying power. When these preparations were crystallized the abnormal activity disappeared, and the crystalline material had the same activity as the usual crystalline pepsin. It seemed possible therefore that this activity was due to the physical state of the precipitate, a possibility which has frequently been suggested in the literature. A more careful study of this material, however, showed that this was not the case, but that the activity was due to a distinct enzyme, "gelatinase," which is extremely active with respect to the liquefaction of gelatin. This "gelatinase" is present in very small quantities in the original material and is completely removed from the crystalline pepsin only with considerable difficulty. The conclusion that the abnormal activity is due to this "gelatinase" depends upon the fact that the abnormally active material cannot be obtained from crystalline pepsin which has been crystallized four or five times. In addition it was found that the activity of the crystalline pepsin could be destroyed in slightly alkaline solution while the gelatin liquefying power of the amorphous preparations was only slightly reduced. This accounts for the occasional abnormal results which were obtained in the study of the alkali-inactivation of crystalline pepsin.

A method has been worked out for the purification of the "gelatinase" and a small amount of protein material has been obtained which was about 450 times as active as the crystalline pepsin as measured by the liquefaction of gelatin, or about 1,000 times as active as the crude pepsin preparation. The activity of this material could not be increased by any method tried, and solubility measurements gave an indication that it might be a pure protein. Owing to lack of material, however, no conclusive results were obtained as to the final purity of the preparation.

A study has been made of the activity of this material in effecting the hydrolysis of gelatin, casein, egg albumin, and edestin as measured by the change of viscosity, increase in formol titration, and production of non-protein nitrogen. The activities of the crude pepsin and crystalline pepsin have also been determined in the same way. The "gelatinase" is considerably less active than the crystalline pepsin in all respects except in the hydrolysis of gelatin. It is three or four times as active as crystalline pepsin in the hydrolysis of gelatin as measured by the formol titration, and 450 times as active as measured by the change of viscosity. It is possible that the activity on proteins other than gelatin is due to small amounts of the crystalline pepsin present in the "gelatinase" preparations. On the other hand, crystalline pepsin preparations which have apparently been entirely freed from the "gelatinase" still possess the power of digesting gelatin.

EXPERIMENTAL RESULTS

Separation from Crystalline Pepsin.—The abnormal activity with gelatin was originally noted when the protein fraction from crude pepsin preparations was dissolved with dilute alkali and partially precipitated with acid. When the precipitate obtained in this way was fractionated by repeated partial precipitation the gelatin-liquefying power increased, and this process could be apparently repeated indefinitely. The proteolytic power as shown with other proteins also increased at first, remained constant after four or five precipitations, and eventually decreased. If the same process be carried out with crystalline pepsin which has been crystallized once or twice, a small quantity of abnormally active amorphous material can be obtained after five or ten partial precipitations. If, however, the crystalline pepsin has been

recrystallized four or five times very little or none of the abnormally active material can be obtained.

Separation by Alkali Inactivation.—If solutions of crude pepsin or of once or twice crystallized pepsin are brought to pH 9.5 or pH 10 for a few minutes and then acidified, the solution still liquefies gelatin more or less rapidly, while if the crystalline pepsin has been recrystallized several times the gelatin-liquefying power is completely lost at pH 9.5 in a very short time. This procedure serves therefore as a

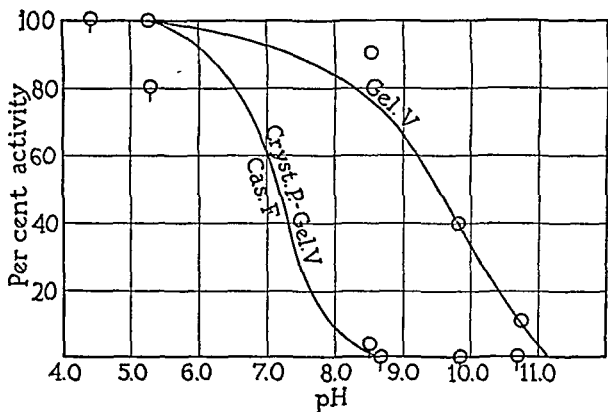


FIG. 1. Loss of activity of crystalline pepsin and crude "gelatinase" at different pH, as measured by the liquefaction of gelatin and by the digestion of casein.

sensitive test for the presence of the "gelatinase." A comparison of the loss of activity of four-times recrystallized pepsin and of a "gelatinase" fraction is shown in Fig. 1. The figure shows that the loss in activity of the crystalline pepsin as measured by loss in gelatin-liquefying power or by the hydrolysis of casein is about the same and is practically complete at pH 9. The "gelatinase" fraction, on the other hand, is less than half inactivated under the same conditions and retains a considerable part of its activity at pH 10.

Preparation of "Gelatinase."—The repeated precipitation at pH 3, mentioned above, leads to increasingly active preparations; but it

TABLE I
Preparation of "Gelatinase"

	Fraction no.
1,000 gm. Parke, Davis pepsin U. S. P. 1:10,000 + 800 ml. H ₂ O + 800 ml. M/1 H ₂ SO ₄ + 1,600 ml. saturated MgSO ₄ .	
Filter. Filtrate discard.	
Ppt.	1
Ppt. 1, stir with $\frac{1}{2}$ volume water and M/2 NaOH stirred in until clear solution (pH <5.0). N/2 H ₂ SO ₄ stirred until pH = 3.0. Stand at 6°C. for 18 hrs.	
Filter. Filtrate.	F ₁
Stand at 6°C., 4 to 6 days, ppt. forms. Filter and discard filtrate.	
Ppt.	P ₂ a
Ppt. Dissolve at 37°C. with $\frac{1}{2}$ volume H ₂ O and M/2 NaOH. Crystallize.	
Filter. Ppt. Crystalline pepsin, recrystallize 3 times	3 C
" 4 "	4 C
Filtrate. Add $\frac{1}{3}$ to $\frac{1}{2}$ volume saturated Na ₂ SO ₄ .	
Filter. Filtrate.	F ₂
Ppt., combine with ppt. P ₂ , dissolve with M/10 pH 5.0 citrate buffer and dilute to 5.0 mg. N/ml. Precipitate by addition $\frac{1}{2}$ to $\frac{1}{2}$ volume saturated Na ₂ SO ₄ , filter and repeat precipitation until ppt. has activity of about 5×10^4 [PU] _{gm. N.} ^{gel. 5}	P ₄
(Combined filtrates from this process may be titrated to pH 3.0 and treated as F ₂)	
P ₄ , dissolve in H ₂ O and dilute to 10 mg. N/ml. Titrate to pH 9.0 (faint pink to phenolphthalein).	
Titrate to pH 4.0 after 1 to 2 min.	P ₇
Centrifuge and wash with 0.002 N H ₂ SO ₄	
Supernatant and washings, discard.	
Ppt., stir with water to thick paste and add 4 volumes per cent P ₃ solution.	
Dialyze against 0.001 HCl at 20°C. until clear solution (3 to 4 days)	P ₉
P ₉ , precipitate by addition saturated Na ₂ SO ₄ solution.	
Filter. Ppt., dissolve with M/10 pH 5.0 citrate buffer and dilute so that solution contains about 5 mg. N/ml. Precipitate by addition of Na ₂ SO ₄ . This fractional precipitation is continued until no further increase in activity is shown by precipitate.	P ₁₀

soon became evident that no appreciable amount of material could ever be obtained in this way, owing to the cumulative losses in the

course of the several hundred precipitations required to reach the maximum activity. A large number of attempts were made to devise a rapid and efficient method of separation of the "gelatinase," without any success. No sharp separation could be found but a method was eventually worked out which although laborious allowed the preparation of an extremely active fraction with very little loss in activity. Most of the crystalline pepsin was removed by crystallization. The filtrate from the crystallization was precipitated by the addition of acid and the resulting precipitate dissolved with weak alkali and reprecipitated with acid. This process was repeated until the gelatin-liquefying power had been increased five to ten times. The material was then dissolved in water, brought to pH 9 and then acidified.

TABLE II
Reactivation of "Gelatinase"

100 ml. P_3 solution (8 mg. N/ml.) titrate to pH 11.5 + NaOH. Titrate to pH 7.0 + HCl. Dilute with M/100 citrate buffer of different pH. Stand at 22°C. for 24 hrs.

pH.....	4.0		5.3		5.6		5.9		6.2		
Final concentration, mg. N/ml.....	3	1	3	1	3	1	3	1	3	1	
Percentage original activity recovered.....	<0.1	<0.1	2	6	2	5	10	5	15	5	12

fied. The pepsin protein is denatured at pH 9.0 and precipitates on acidification, carrying with it the "gelatinase." A small amount of active pepsin is added to this precipitate and the mixture dialyzed against 1/100 M hydrochloric acid. Under these conditions the denatured pepsin protein is quite rapidly digested and passes out through the membrane leaving the "gelatinase." The solution obtained in this way is then fractionated by precipitation with sodium sulfate. About 30 per cent of the total gelatin-liquefying activity present in the original material may be obtained by this method. Details of the method of fractionation are shown in Table I.

The final fraction is a protein and loses its activity by heating or by the addition of strong alkali. Part of the activity can be recovered after alkali inactivation by titrating to pH 6. The results

TABLE III
Specific Activity of Various Pepsin Fractions. $[PU]_{gm\ N}$

Pepsin fraction.....	Method	Parke, Davis	Crystal-line	P_3	P_6	P_7 (alkali inact.)	P_{10}	"Gelatinase"
Gelatin	2.5 per cent, pH 5.0 -viscosity $[PU]_{gm\ N} \times 10^{-5}$ 5 " " pH 2.5 " " "	0.056 0.16	0.13 0.44	0.40 1.0	0.77 1.0	0.64 0.5	16 17	60
Casein	Formol $[PU]_{gm\ N}$ 5 " " pH 2.5 -viscosity $[PU]_{gm\ N} \times 10^{-5}$ + " " " " "	0.4 2.8 0.3	1.1 12 1.5	0.6 8.5 2.0	1.0 6 1.2	0.4 0.1 0.01	1.7 3.5 0.5	
Milk	Formol $[PU]_{gm\ N}$ NPN	16 100	90 440	60 290	40 200	0.8 6.6	20 120	
Egg albumin	Rennet $[PU]_{gm\ N} \times 10^{-7}$ 5 " " pH 2.0 -viscosity $[PU]_{gm\ N} \times 10^{-5}$ + " " " " "	5.3 0.5 0.001	28 1.4 0.01		10	0.22	7.40 0.3 0.002	
	Formol $[PU]_{gm\ N}$	0.002						
	NPN	17 25 44	50 60 120				20 30	
Edestin	pH 1.9 -viscosity $[PU]_{gm\ N} \times 10^{-5}$ 5 " " " " " Formol $[PU]_{gm\ N}$ NPN	30 1.2 25 30 70	5.7 80 400				1.8 35	

of an experiment in which about 10 per cent of the activity was recovered are shown in Table II. The result is probably due to the reversal of denaturation of the protein.

Activity of Various Fractions

The activities of some of the fractions obtained in the course of the preparation with casein, egg albumin, edestin and gelatin were determined, as was also the effect on the rate of clotting of milk. Owing

TABLE IV

Activity of Crude Pepsin and of "Gelatinase" as Per Cent of the Activity of Crystalline Pepsin

Protein	Method	Parke, Davis pepsin	"Gelatinase" P ₁₀
Gelatin	2.5 per cent, pH 5.0 —viscosity	40	12,000
	5 " " pH 2.5 " "	36	3,800
	Formol	36	150
Casein	5 " " pH 2.0 —viscosity	23	30
	+ " "	20	35
	Formol	18	22
	NPN	23	27
	Rennet	19	27
Milk Egg albumin	5 " " pH 2.0 —viscosity	35	21
	+ " "	20	20
	Formol	40	40
	NPN	30	25
	Edestin	21	32
Edestin	5 " " pH 1.9 —viscosity	30	42
	Formol	30	42
	NPN	18	

to the great difficulty in preparing the most active material the determinations were not carried out with this fraction but only with the fraction having about $\frac{1}{4}$ the maximum activity so far obtained. The results of these determinations are shown in Table III in which the activity per gram of nitrogen has been tabulated against the various fractions. The table shows that the activity of the crystalline pepsin is about five times that of the original material in every respect except the hydrolysis of gelatin. In this respect it is $2\frac{1}{2}$ times as active as the original material. The amorphous precipitates obtained from the mother liquor of the crystallization are less active than the crystals

TABLE V
Activity of Pepsin Fractions by Different Methods Expressed as Per Cent of the Activity Measured by the Increase in NPN with Casein

Pepsin.....				"Gelatinase" P ₁₀	
Protein	Method	Parke, Davis			Crystalline	
Gelatin	2.5 per cent, pH 4.7 —viscosity	0.56 × 10 ⁴			0.3 × 10 ⁴	130 × 10 ⁴
	5 " " pH 2.5	1.6 × 10 ⁴			1.0 × 10 ⁴	140 × 10 ⁴
Casein	Formol	0.4			0.25	1.4
	5 " " pH 2.2 —viscosity	28 × 10 ⁴			27 × 10 ⁴	29 × 10 ⁴
	+ " "	3 × 10 ⁴			3.3 × 10 ⁴	4 × 10 ⁴
	Formol	16			20	16
Milk Egg albumin	NPN	[100]			[100]	[100]
	Rennet	5.3 × 10 ⁷			6.3 × 10 ⁷	6 × 10 ⁷
	5 " " pH 2.2 —viscosity	5 × 10 ⁴			3.2 × 10 ⁴	2.6 × 10 ⁴
	+ " "	0.02 × 10 ⁴			0.02 × 10 ⁴	0.017 × 10 ⁴
Edestin	Formol	23			12	16
	NPN	36			27	25
	5 " " pH 1.9 —viscosity	12 × 10 ⁴			13 × 10 ⁴	15 × 10 ⁴
	Formol	20			18	29
	NPN	70			90	

in their action on casein edestin and egg albumin solutions, but more active in their action on gelatin. As the fractionation continues this difference becomes more marked.

When the material is inactivated by alkali all but a few per cent of the activity as measured by the digestion of casein, edestin, or egg albumin is lost, while only about $\frac{1}{2}$ of the gelatin activity is lost. After dialysis and further precipitation the specific activity as measured by coagulation of milk or by the effect on casein, egg albumin, or edestin solutions decreases until it is about the same as that of the original crude material, while at the same time the gelatin-hydrolyzing power increases rapidly, especially when measured by the change in viscosity. These changes may be seen more clearly from Table IV, in which the results are expressed as *per cent* of the activity of the crystalline pepsin.

In Table V the results have been calculated as *per cent* of the activity as measured by the increase in non-protein nitrogen for casein. That is, the activity as determined by the increase in non-protein nitrogen for casein is taken as 100 and the activity as measured in other ways expressed as *per cent* of this value. The table shows that the most marked change in the relative activity as expressed as per cent of casein digestion again occurs in the case of gelatin. In this case the activity of the original preparation as measured by the change in viscosity is about 1×10^4 per cent of that as measured by casein digestion, while in the "gelatinase" fraction it is about 130×10^4 per cent.¹ This difference in the properties of the "gelatinase" and crystalline pepsin may be shown more clearly by plotting the increase in formol titration against the viscosity of the solution, as was done in the study of the changes in gelatin solutions caused by pepsin digestion (2). The result of an experiment done in this way is shown in Fig. 2. The figure shows that the "gelatinase" causes a relatively enormous decrease in the viscosity of the gelatin solution with a very slight increase in formol titration, while in the case of

¹ Since the activity units for the various methods have different dimensions, these percentage figures have no physical significance but are merely comparative. The activity as determined by any one method, however, is commensurable and may be compared directly, so that in this case the percentages are a measure of the relative activity by this method.

the original material or the crystalline pepsin there is a much larger increase in formol titration for the same decrease in viscosity. In the case of the "gelatinase" the viscosity is decreased nearly 50 per cent before any change in the formol titration can be detected by the method used here, which means that there is a very large change in the physical condition of the gelatin solution for a very small chemical change, or else that there are chemical changes in the gelatin which

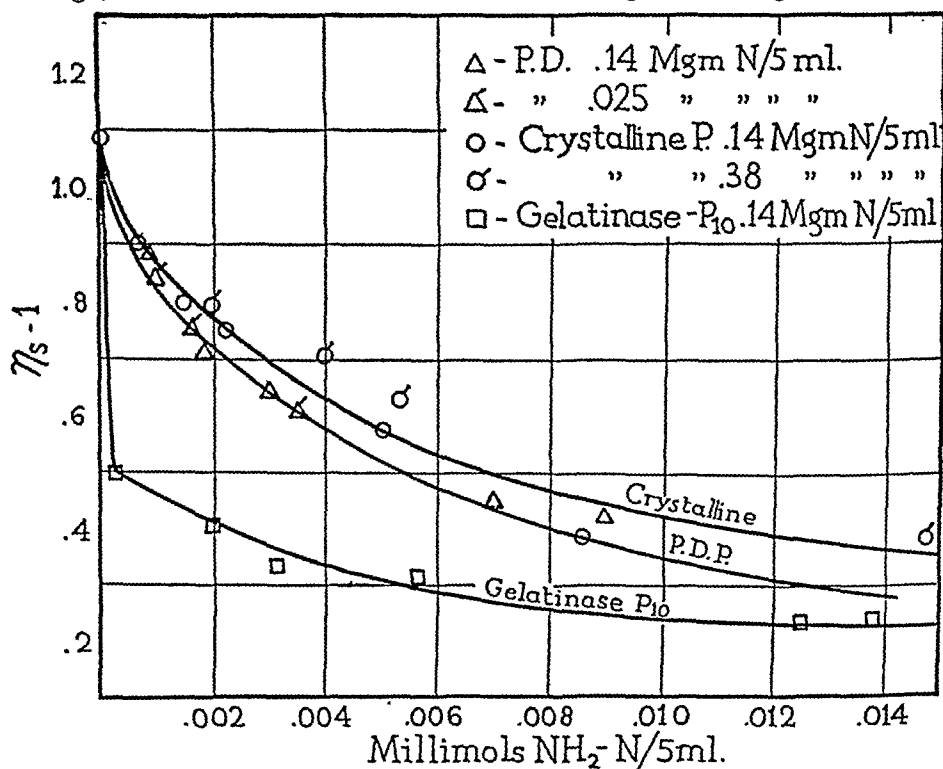


FIG. 2. Change in viscosity compared to the increase in amino nitrogen of Parke, Davis pepsin, crystalline pepsin and "gelatinase."

do not result in the liberation of free amino groups. It is probable that the chemical changes start at once and that they could be detected by the change in conductivity or some other more sensitive method. On the other hand, it follows from the structure of gelatin solutions as suggested by Kunitz and the writer (3) that a very slight amount of hydrolysis, especially if it were restricted to the insoluble fraction of gelatin, might cause extremely great changes in viscosity.

These experiments indicate that the activity of the "gelatinase"

fraction on proteins other than gelatin is due to the presence of some pepsin, and that if this were removed the "gelatinase" would act only on gelatin. If this were the case it would be expected that repeated alkali-inactivation would destroy the activity on the other proteins. If the "gelatinase" is brought to pH 9.5 there is a considerable loss of activity with casein or edestin, but repetition of this procedure, causes only a slight additional loss. The result of an experiment of this kind is shown in Table VI. It shows that part of the casein- and edestin-digestive activity is more unstable than the gelatin-liquefying activity, but that some of the activity for casein and edestin has about the same stability as that for gelatin.

TABLE VI
Alkali Inactivation of P₁₀

	Per cent activity		
	Gelatin V.	Casein V.	Edestin V.
P ₁₀ solution, 0.10 mg. N/ml.....	100	100	100
Titrate to pH 9.5 for 1 min. and then to pH 5.0.....	100	35	37
Titrate to pH 10.0 for 1 min. and then to pH 5.0.....	90	30	27

Changes in the Properties of Casein Solutions.—The addition of pepsin to acid casein solutions causes at first a very rapid decrease in viscosity, followed by an increase. Table V shows that the "gelatinase" fraction causes the viscosity to increase relatively more rapidly than does the crystalline pepsin or the Parke, Davis pepsin.

Experiments of a similar kind have been performed by Holter (4) who found that various pepsins differed in the way in which they affected the viscosity of casein as compared to their effect as measured by the titration of carboxyl groups. Holter plotted the increase in acid groups against the changes in viscosity and obtained characteristic curves for the various pepsin preparations. A series of experiments were carried out therefore in which the change in viscosity, the change in formol titration, and the increase in nitrogen not precipitated with trichloroacetic acid (NPN) were determined. The

results of these determinations are shown in Fig. 3, in which the specific viscosity of the solution has been plotted against the milli-equivalents of non-protein nitrogen per 5 ml. of solution. The figure shows that the "gelatinase" fraction causes a greater increase in viscosity for the same increase in non-protein nitrogen than does

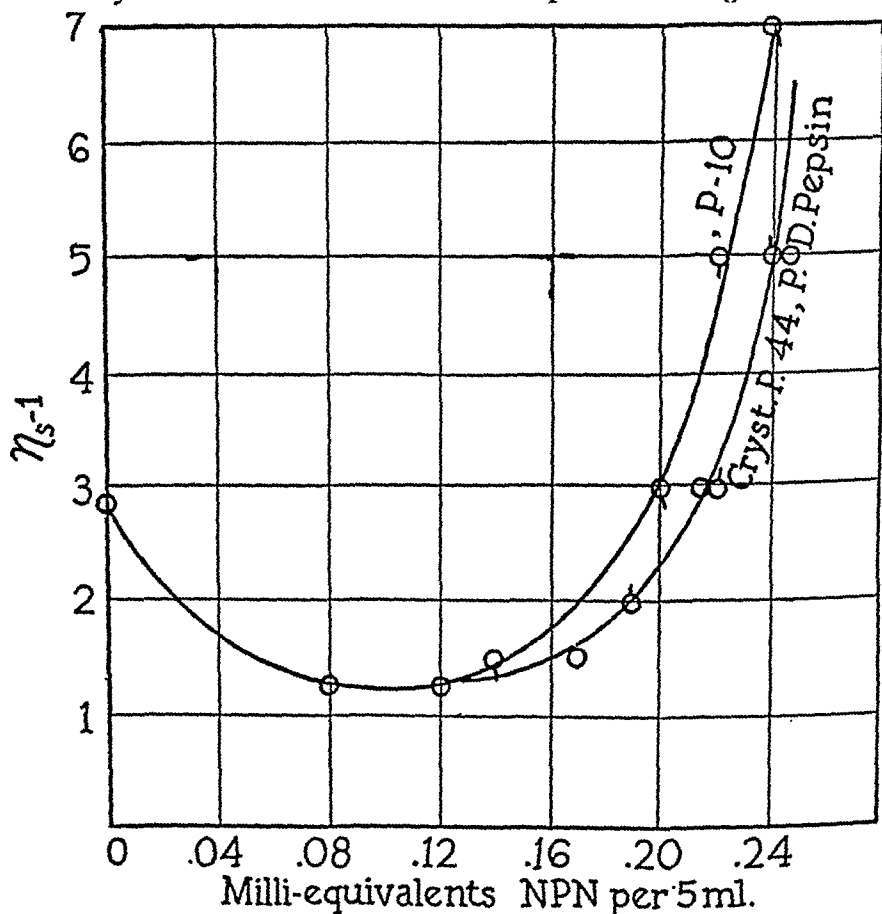


FIG. 3. Change in viscosity as compared to formation of non-protein nitrogen with crystalline pepsin, Parke, Davis pepsin and "gelatinase."

the crystalline pepsin. The original material gives results very close to those for the crystalline pepsin. The formol titration gave similar results, but the measurement is not as reliable. These curves are similar to those obtained by Holter for fractions separated by means of aluminum hydroxide, and indicate the possibility that the "gelatinase" fraction obtained in these experiments is the same as that

obtained by Holter by the use of aluminum hydroxide. Holter, however, states that a fraction may be obtained from crystalline pepsin, which shows the same peculiarity, by means of aluminum hydroxide. As was stated before, the "gelatinase" may also be prepared from crystalline pepsin after one or two crystallizations but not after four or five crystallizations.

These experiments were not carried out in greater detail. It will be noted that the viscosity of egg albumin also decreases and then increases while the viscosity of edestin solutions decreases only. No

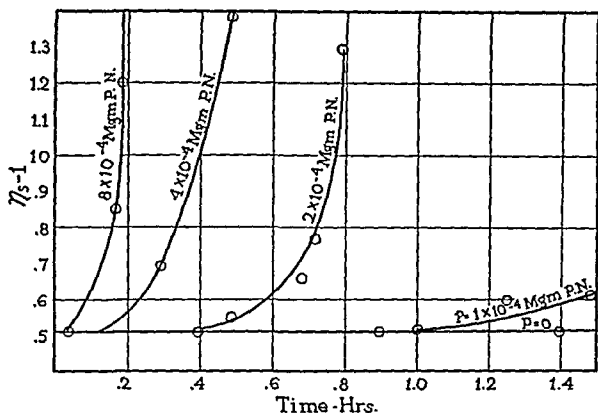


FIG. 4. Change in viscosity of milk with various concentrations of crystalline pepsin.

indication of an increase in the viscosity of edestin solutions could be found even with very high concentrations of pepsin.

The activity of the various fractions as measured with egg albumin and edestin is about the same when expressed as per cent of the casein digesting activity (Table V).

Activity Units.—In the preceding paper (1) the activity was expressed as milliequivalents of amino nitrogen liberated per minute under standard conditions. The unit of activity as measured by change of viscosity was based on this measure-

GELATIN-LIQUEFYING ENZYME IN CRUDE PEPSIN

ment of the increase in amino nitrogen. Since in the present work the activity as measured by the change of viscosity is found to vary independently of the activity as determined in other ways, it is necessary to have an independent unit for the activity as measured by viscosity changes. Since the percentage changes in viscosity of protein solutions of various concentrations are nearly independent of the protein concentration, it seems better to define the activity in terms of percentage change in the additional viscosity rather than absolute change. The activity units used in this paper are defined as follows:

The quantity of enzyme which, when added to 5 ml. protein solution, will cause a change of 1 per cent per minute at 35.5°C. in the additional viscosity.

$$a. \text{ Negative} = [\text{PU}]^{\text{Protein V.}} = \frac{100 (T_o - T_i)}{t_{\text{min.}} (T_o - T_{\text{H}_2\text{O}})}$$

$T_{\text{H}_2\text{O}}$ = Time outflow of water.
 T_o = " " " solution at time 0.
 T_i = " " " " " "
 t = Elapsed time in minutes since addition of enzyme.

$$b. \text{ Positive} = [\text{PU}]^{\text{Protein V}^+} = \text{Per cent change in additional viscosity of protein solution per minute, at 35.5°C. when viscosity has returned to its original value.}$$

$$\text{Non-Protein N} = \text{NPN} = [\text{PU}]^{\text{Protein NPN}} = \text{Milliequivalents nitrogen not precipitated by 10 per cent trichloroacetic acid per 5 ml. solution per minute at 35.5°C.}$$

$$= [\text{PU}]^{\text{Protein F}} = \text{Milliequivalents amino nitrogen per 5 ml. solution liberated per minute at 35.5°C.}$$

Formol

$$= [\text{PU}]^{\text{R}} = \frac{100}{t_{\text{min.}}} \text{ Per cent change in additional viscosity of standard milk, calculated from the point at which the additional viscosity has doubled.}$$

Renner Action

$$T_{\text{min.}} = \text{Elapsed time at which } \Delta\eta = 2 (\eta_o - 1)$$

The negative viscosity measurements were made at short intervals with an Ostwald viscosimeter and the values determined in this way plotted against the elapsed time. A line is drawn through these points and the initial change in viscosity per minute determined from the slope of this line. The measurements are confined to the first 5 per cent change in viscosity, and in this range the change in viscosity per unit of time is constant except in the case of egg albumin. In all cases the determinations were made with very low concentrations of enzyme and the specific activity was independent of the quantity of enzyme used.

The positive viscosity rate was determined by plotting the viscosity against the elapsed time, and determining the slope of this curve at the point at which the viscosity had returned to its original value.

The increase in formol titration and non-protein nitrogen was determined by analyzing 5 ml. samples after 1, 2 and 3 minutes and the initial rate determined by interpolation from the curves.

The rennet activity was determined by plotting the change in viscosity against the elapsed time and determining by interpolation the time at which the increase in viscosity was equal to the original specific viscosity. 100 times the reciprocal of this time in minutes is taken as equal to the number of pepsin units as measured by rennet action.

EXPERIMENTAL METHODS

The experimental procedure and the protein solutions used in these experiments were in general the same as those described in the preceding paper (1).

The rennet action, however, was determined this time by following the changes in viscosity of a standard solution of commercial milk powder. This was prepared by dissolving 10 gm. of "Klim" milk powder in water and making up to 100 ml. 10 ml. of a molar solution of pH 5 acetate buffer is then added. 0.2 ml. of the pepsin preparation is added to 5 ml. of this standard milk and the change in viscosity at 35.5°C. determined.

The changes in the viscosity of isoelectric gelatin solutions are the most reliable and accurate. The changes in the viscosity of the solutions of edestin and casein are also accurate and reliable for comparative determinations but differ somewhat with various protein preparations. The determinations with egg albumin are the least satisfactory, in that all the methods show a relatively rapid change at the very beginning of the reaction which then becomes very much slower. Considerable variation was noted also with different egg albumin solutions, and the results with this protein may not be correct except as to their order of magnitude.

SUMMARY

A protein fraction has been isolated from crude pepsin preparations which is about 400 times as active as crystalline pepsin in the liquefaction of gelatin.

The activity as measured by the digestion of casein, edestin or egg albumin is less than that of crystalline pepsin.

It is more resistant to alkali than the crystalline pepsin.

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STUDIES ON MILK SECRETION

THE INFLUENCE OF INANITION

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Milk secretion may be looked upon as a two-step process. The first step consists in the passage of the materials for the formation of milk from the blood stream into the gland cells. In the second step these materials are organized by the gland cells into the constituents of milk, when they are then passed into the alveolar spaces of the udder. The question arises as to what may be the motivating causes which bring about these changes.

The early contributions to this subject were largely those made by the histologist studying the changes which he observed in the tissue of the mammary glands. These histological observations have been markedly influenced in their interpretation by the supposed modes of secretion of two other types of glands, the sebaceous and the salivary. This work led to three major hypotheses to account for milk secretion by the mammary glands:

1. The cells of the glands break loose bodily and disintegrate in the alveoli to form the milk solids.

2. A portion of the cells toward the alveoli become loaded with solids, are broken from their base, and disintegrate to form the constituents of milk.

3. The cells of the mammary glands secrete the materials of the milk solids without the cells breaking down.

The first theory has largely been discredited because as Heidenhain (1)¹ pointed out this theory would call for a cell destruction and replacement of as high as five times the number of cells in the udder in a single day; a rate of cell replacement which was not observed.

¹ The literature citations are given in the paper following.

The second theory, suggested by Langer and supported by Heidenhain (1), Steinhaus (2), and Brouha (3), lays its foundation on histological evidence. The gland cells are represented as lengthening out into the lumen of the alveoli. The projecting ends of these cells become loaded with nutrients similar to those of the milk solids, these projecting ends disintegrate and allow the solids to escape. The basal portion of the cell, including the nucleus, is left to rebuild the cell and repeat the process.

The third theory lays its stress on analogy with the other secretory glands. In support of this thesis Bertkau (4) says that the disintegration of the udder cells which other observers have claimed visible in mammary gland tissue is due to imperfect fixation and that when the tissue is properly fixed no necrobiosis of any kind appears. This theory is further supported by the study of the individual constituents of milk and their variations during the day and the lactation period (Gowen (5)).

As indicated above, milk secretion may be looked upon as a two-step process,—the passage of the precursors of milk from the blood into the secreting cells and their subsequent passage from these cells into the milk cisterns. The first two theories cited only visualize the last phase of this process. The third theory accounts for the whole process if we assume with Simms (6) that the constituents on the two sides of the cell membranes have unlike affinities for salts, etc., or secondly are differentially permeable.

As blood is the common carrier for the materials utilized in metabolism, it is natural to turn to it as the source of the precursors of milk solids. Thus far, the most fruitful method of analyzing what these precursors of the milk solids may be, has been the comparing of the composition of the blood which is entering the mammary gland with the composition of that coming away from it. This method of approach, first devised by Kaufmann and Magne (7), has shown that in all probability dextrose is the source of lactose in milk. The observations on which this conclusion is based have been substantiated by Cary (8) and also by the present writers (unpublished).

Meigs, Blatherwick, and Cary (9) have utilized the same methods to indicate the origin of milk fat. The reasoning is based on their experimental observations as follows:

The blood on entering the udder carries more phosphatides than that leaving the udder. The milk entering the udder carries less inorganic phosphate than that leaving the udder. From these observations and the difference in the composition of milk compared to that of blood with regard to phosphorus, they draw the conclusion that the mammary gland in butter fat secretion takes phosphatides from milk, converts them into fat, and returns as inorganic phosphorus the excess of this element derived in breaking down and synthesizing the milk fat from the phosphatide.

Cary following the same line of approach showed that the blood entering the udder contains more amino nitrogen than the blood coming away from the udder. From this fact and the work on metabolism of proteins he draws the conclusion that the proteins of milk are derived from the amino acids of blood.

In studying the composition of the fat deposited in the udders of cows in milk secretion, as contrasted with the udders of such cows in the dry period, the fats found in the actively secreting udder were found to be partly metabolized toward the characteristic fats of milk. The fat in the dry udder, on the other hand, does not show this step even though it contains proportionately much more fat than the actively secreting udder. The secretion of milk-fat appears as a distinct two-step process. The blood brings to the udder materials for deposition in the udder of fats comparable to other body fats. These fats are then converted by the udder, somewhat in advance of their need for milk secretion, into the fats characteristic of milk (Gowen and Tobey (10), Peterson, Palmer, and Eckles (11)).

These hypotheses and the facts just cited, however imperfect they may be, furnish an important approach to the problem of the mechanism of milk secretion. They illustrate, however, the desirability of other methods of attack. In the summers of 1925, 1926, and 1927, the writers attempted such an approach.

In view of the fact that most of the original constituents of milk find their origin in the blood stream, it would follow that if any means of altering the chemical characteristics of the blood stream could be found it would thereby be expected to affect the characteristics of the milk secreted. The particular type of alteration observed in the blood compared with the changes noted in the milk would furnish some idea

as to the precursors of the constituents of milk as they are found in the blood. Furthermore, it seemed probable that the changes brought about by altering the blood stream would affect the physical characteristics of the blood stream, such as the osmotic pressures, etc., and by the subsequent alterations in the composition of the milk so produced furnish important facts on the mechanism by which it is normally secreted.

Four means of altering the characteristics of the blood seemed open to us. The first was the change brought about in the blood stream by complete inanition; the second method consisted in the introduction of the hormone of the parathyroid gland with its effect on the calcium of the blood; the third consisted in the introduction of insulin into the animal with the attendant lowering of the blood sugar and the other changes in blood composition; and lastly, it seemed desirable to check the effect of phloridzin on cow's milk rather than on goat's, as had been done by others. The experiment on inanition was also regarded as a control of the experiment on the use of insulin, as at that time it was believed that insulin would not produce its effect without inanition accompanying it. This experiment may be considered first. It may also be combined with the experiment on the action of the parathyroid hormone plus starvation, as the data on this experiment indicate that it is the starvation which produced the observed effects; the parathyroid hormone being without observed effect.

Material and Methods

Our experiment on the inheritance of milk production (11 etc.) included eight cows which were available for the work herein described. The cows ranged in production up to 40 pounds of milk a day and included animals with both high and low butter fat percentages in their normal milk. The animals were all in good condition for milk production, had no tuberculosis, as proved by their clinical tests and autopsy. (The herd is accredited.) The animals were maintained on a ration which by present standards was more than adequate for their milk production. In the initial period the animals passed through the same routine as that which followed for the experimental periods; they were milked, watered, cleaned and, if given food, fed at the same time as during the experimental period.

The first effort was directed toward determining what the normal variation in the milk production at these times really was and the variations in the composition of the milk so produced. In the chart the initial daily milking periods, before the experiment was commenced, represent quite accurately the true milk secretion of the given cow in this period of lactation fed on a production ration.

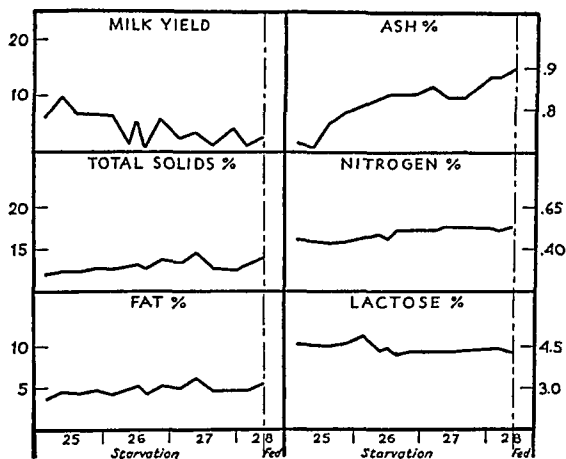


FIG. 1. Cow 47, age 8 years 11 months, 3 months in lactation. Food completely withheld; water given freely. Throughout the amount of material is plotted against time in days.

The total solids, fat, nitrogen, ash, and lactose, were determined by the methods approved by the Association of Official Agricultural Chemists. The calcium was determined by Collip's modification of the Kramer and Tisdall method. Phosphorus was determined by the method of Benedict. Folin's method of blood sugar determination was used and Benedict's method for estimating sugar in urine. The calcium and sugar of the urine appeared in such small amounts with no apparent relation to the other observations that they are omitted

STUDIES ON MILK SECRETION

from the discussion. In the first experiments on two cows, 47 and 141, the milk calcium and phosphorus and blood calcium and dextrose

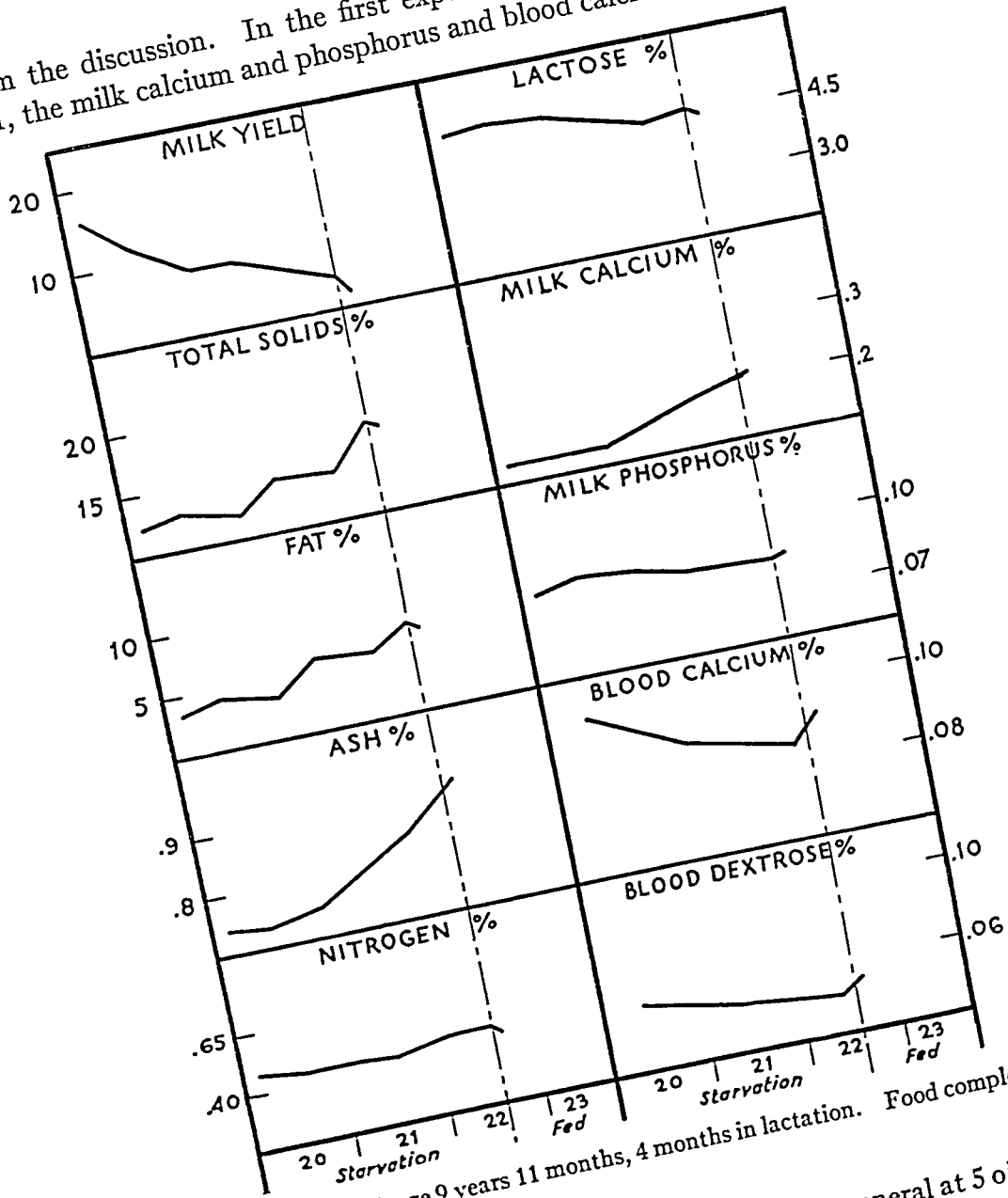


FIG. 2. Cow 47, age 9 years 11 months, 4 months in lactation. Food completely withheld; water given freely. The milking periods were in general at 5 o'clock in the morning and 3 o'clock in the afternoon. In certain cases when

closer intervals were desired the milking periods were 5 and 11 o'clock in the morning and 5 in the afternoon and 11 o'clock in the evening.

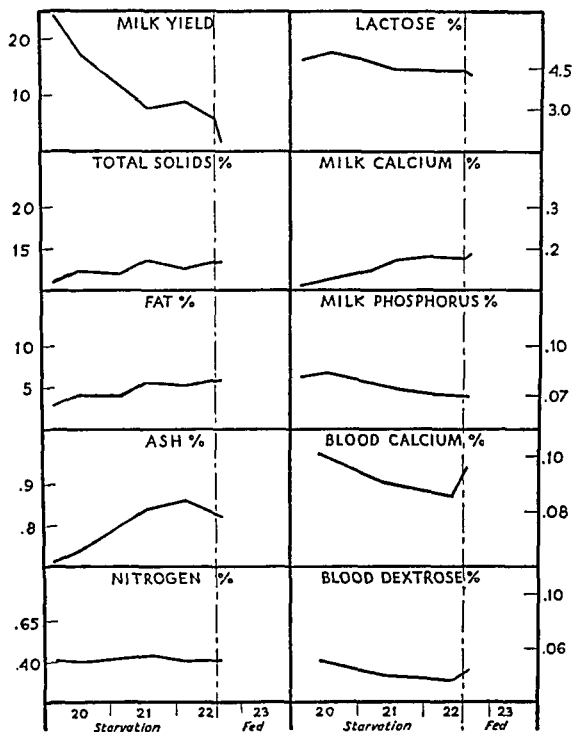


FIG. 3. Cow 52, age 9 years 8 months, 2 months in lactation. Food completely withheld; water given freely.

The Data

The milk yields and percentage composition of the milk and blood are presented chronologically for each cow in Graphs 1 to 13.

The figures are divided into two series of charts, on the left and right hand sides of the page. The upper left hand chart gives the milk yield in pounds plotted against time—duration of starvation. The charts which follow are per cent total solids, fat, ash, and nitrogen of the milk produced; all plotted against duration of inanition. The upper right hand chart is per cent of lactose. Following in sequence

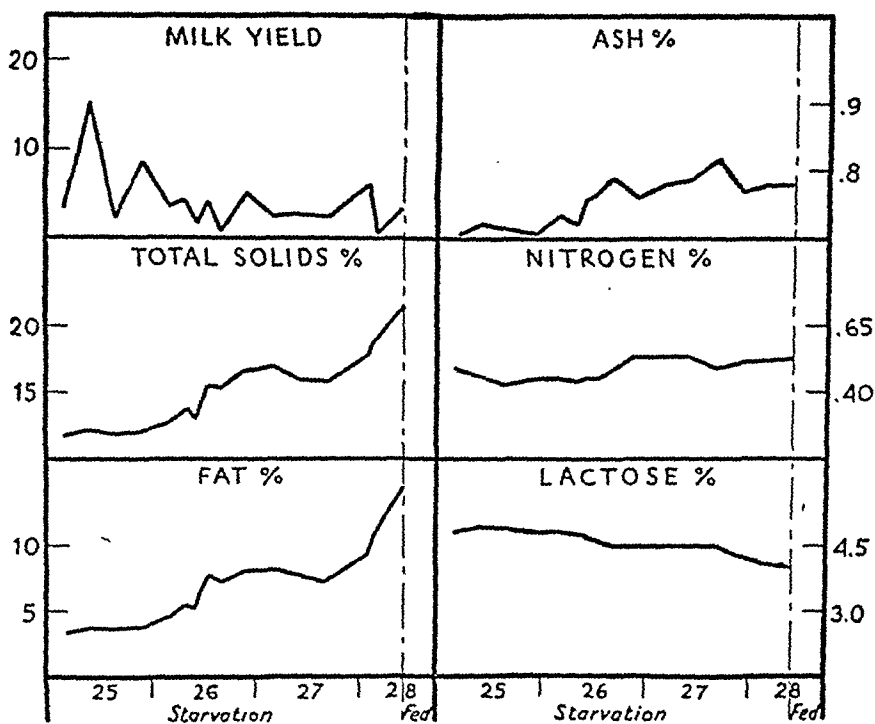


FIG. 4. Cow 141, age 5 years 0 months, 4 months in lactation. Food completely withheld; water given freely.

down the page are per cent of milk calcium, milk phosphorus, blood calcium, and blood dextrose; each plotted against the same chronological scale.

DISCUSSION

Relation between Inanition and Milk Secretion

Figs. 1 to 8, inclusive, bring out certain facts which, while not new to the literature of inanition have as yet not been explained. The cows all exhibit a rapid drop in milk yield almost from commencement

of the inanition period. The rate of this decrease is greater during the first period of inanition, tending to tail off as inanition progresses

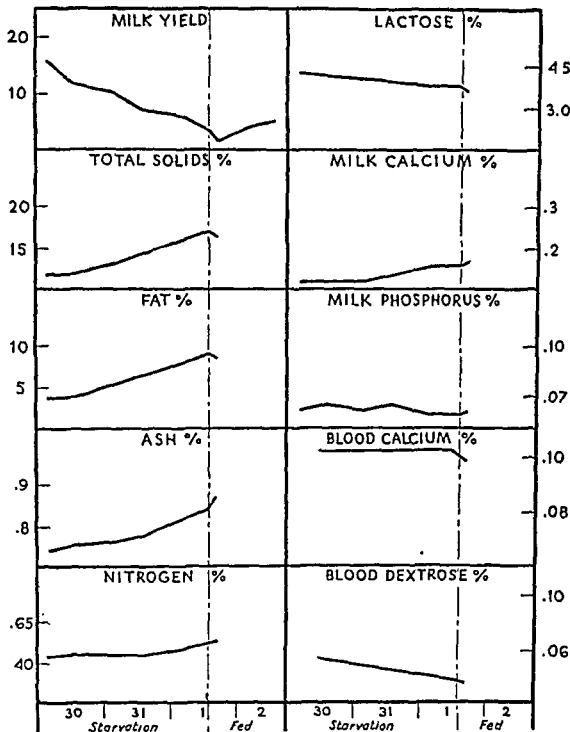


FIG. 5. Cow 141, age 6 years 0 months, 4 months in lactation. Food completely withheld; water given freely.

to an asymptotic value of zero milk production. As the milk production decreases the percentage composition of the solids in the milk

tends to increase rapidly to values which may be two times as great as those ordinarily found within milk. The average increase for the

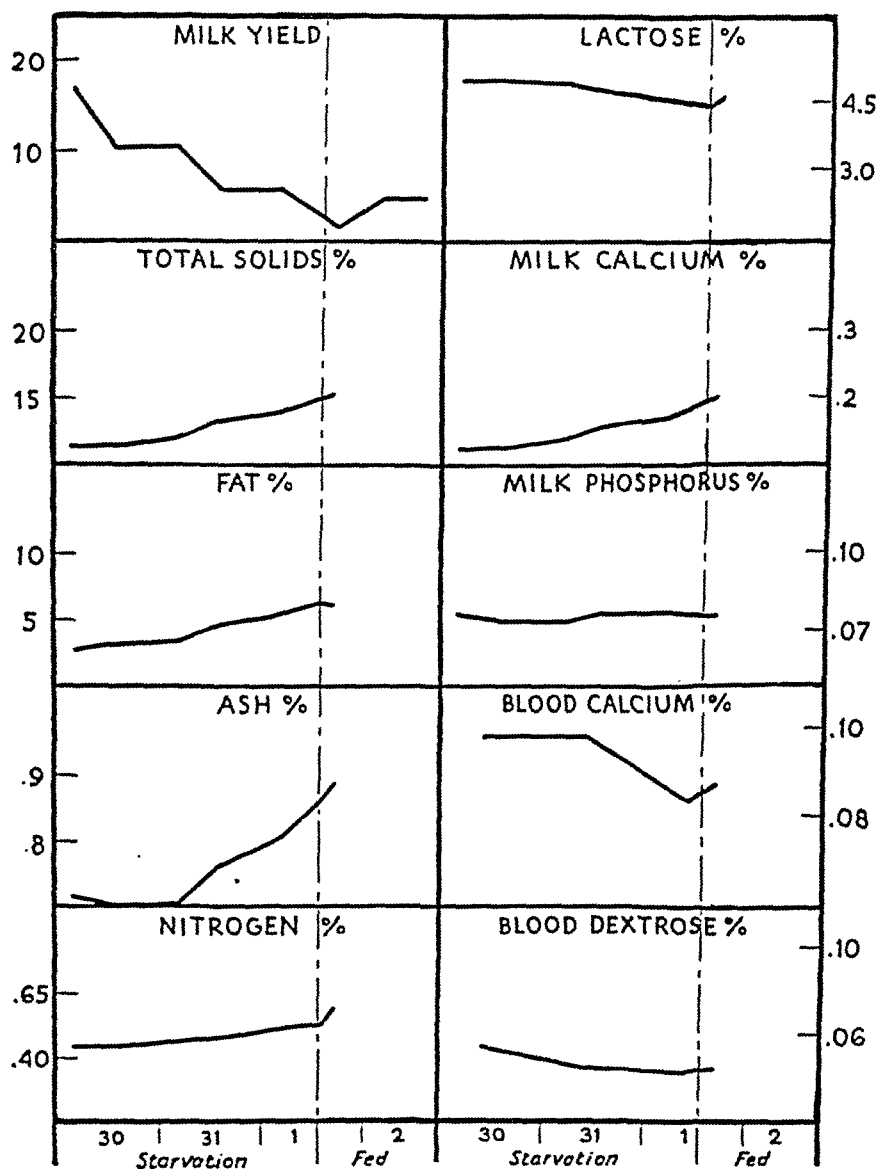


FIG. 6. Cow 212, age 10 years 0 months, 12 months in lactation. Food completely withheld; water given freely.

duration of the starvation period is 1.3 times the customary total solids per cent of these milks. This increase in total solids is very

largely accounted for by the increase in the percentage composition of the butter fat. The butter fat may reach a percentage of 2.5 times

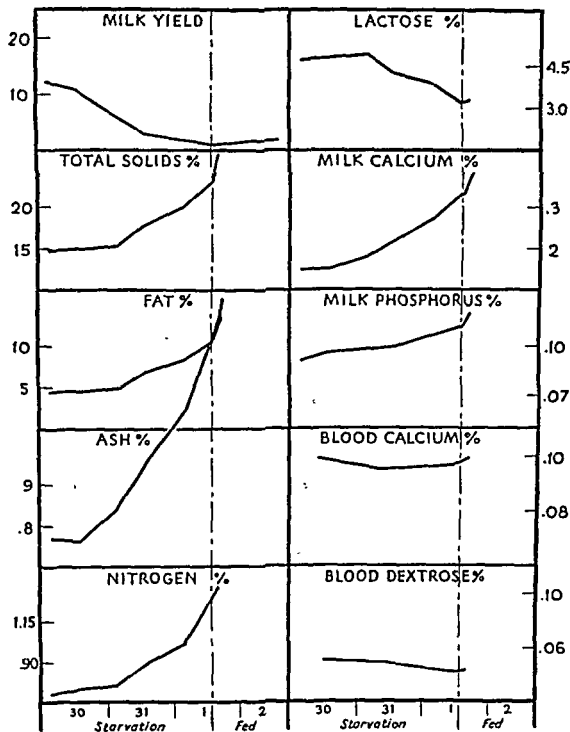


FIG. 7. Cow 213, age 5 years 10 months, 1 month in lactation. Food completely withheld; water given freely.

its normal value. The average increase for the duration of these experiments is 1.9 times the normal value. The ash content of the

STUDIES ON MILK SECRETION

milk also increases in a marked degree. In one case this increase amounted to 1.7 times the ordinary ash content of the milk. The

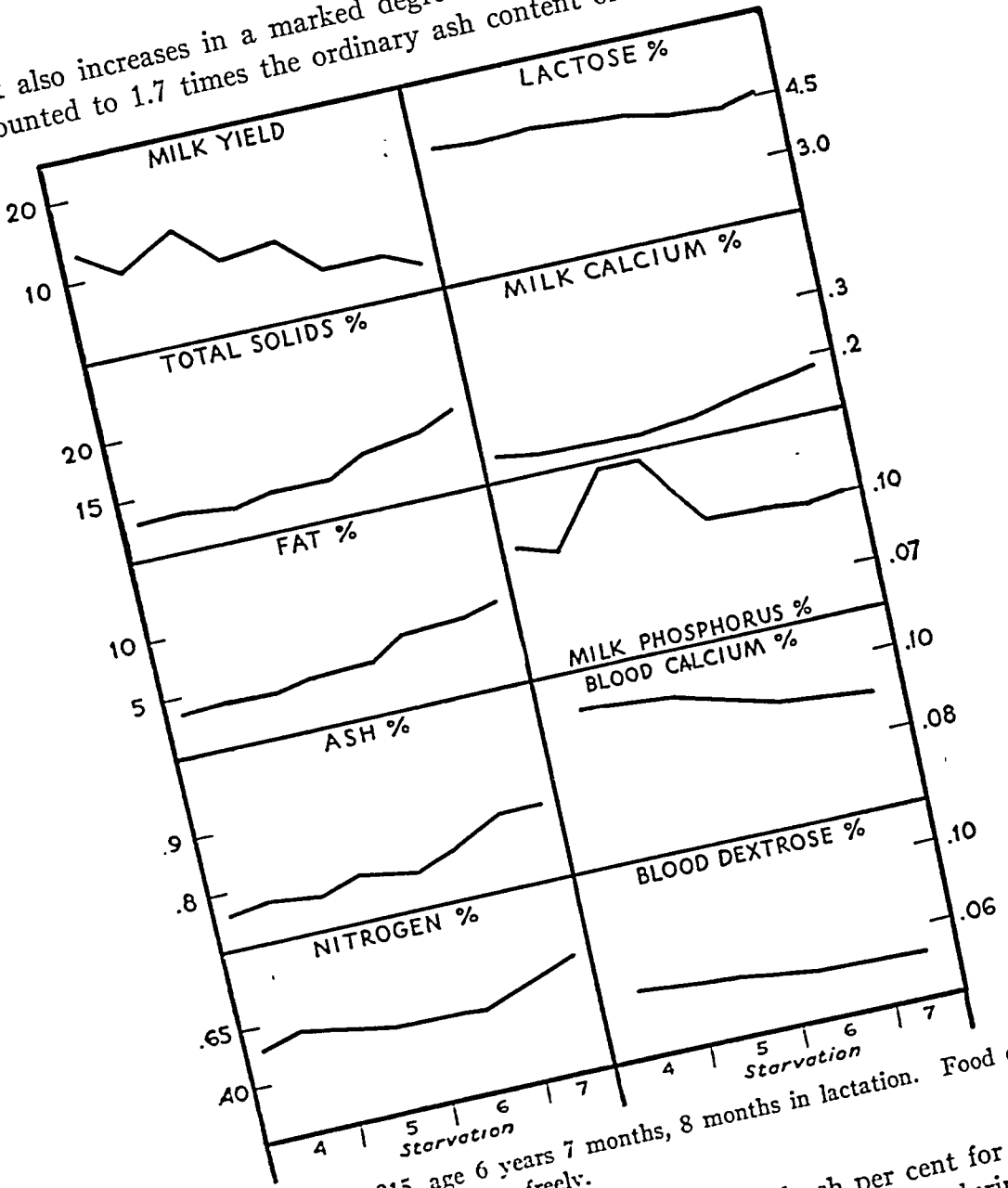


FIG. 8. Cow 215, age 6 years 7 months, 8 months in lactation. Food completely withheld; water given freely.

average gain was about 1.2 times the normal ash per cent for milk. The percentage content of the nitrogen rose but slightly during inanition period for seven of the cows. For the eighth cow it changed

markedly. The nitrogen content of this cow's milk rose to nearly twice its normal value.

The lactose percentage, in contradistinction to the other materials of the milk, showed a distinct decrease in its percentage as the duration of the starvation period increased. This decrease on the average amounted to about one-tenth of the amount of lactose ordinarily found. The milk calcium increased to a somewhat greater extent than did the ash percentage, showing that the ash constituents probably changed in a manner out of proportion to their ordinary content in the ash of milk. The phosphorus content of the milk behaved irregularly but on the whole took a neutral trend throughout the period of inanition.

The blood calcium was found to decrease slightly during the period of inanition. As starvation progressed, the blood sugar was found to decrease.

These results agree with those of Overman and Wright (12) in all essential particulars. For their experiment three cows were available and the inanition period was of longer duration than that noted here. The longer duration of starvation simply extended the effects. The milk yield was reduced to a somewhat lower point, the total solids, fat, protein, and ash, were increased to higher percentages, whereas the lactose was decreased to a markedly lower amount. They do not consider the blood changes. Before considering the mechanism by which these changes are brought about, the data for starvation plus the injection of the parathyroid hormone may be noted. The parathyroid hormone was thought to offer a means of altering the composition of the blood of the cow for another constituent, the calcium. As this constituent plays a large rôle in milk secretion, being particularly associated with the casein, it seemed worth while to try the hormone's effect on milk secretion. With the information which had been gained from the use of insulin, it seemed that the amounts which would be necessary to produce an effect would be relatively high. 200 or 400 units were used at an injection; the material being injected directly into the jugular vein. The parathyroid hormone was the best available. We are indebted to Eli Lilly and Company for it. Unfortunately, the cow takes so much of this hormone that even 1000 units, over the relatively short time of two days, did not alter the composition

of the cow's blood. This was true for both sets of animals; one set with the hormone accompanied by complete inanition, the other set with the hormone accompanied by feeding the normal ration. The

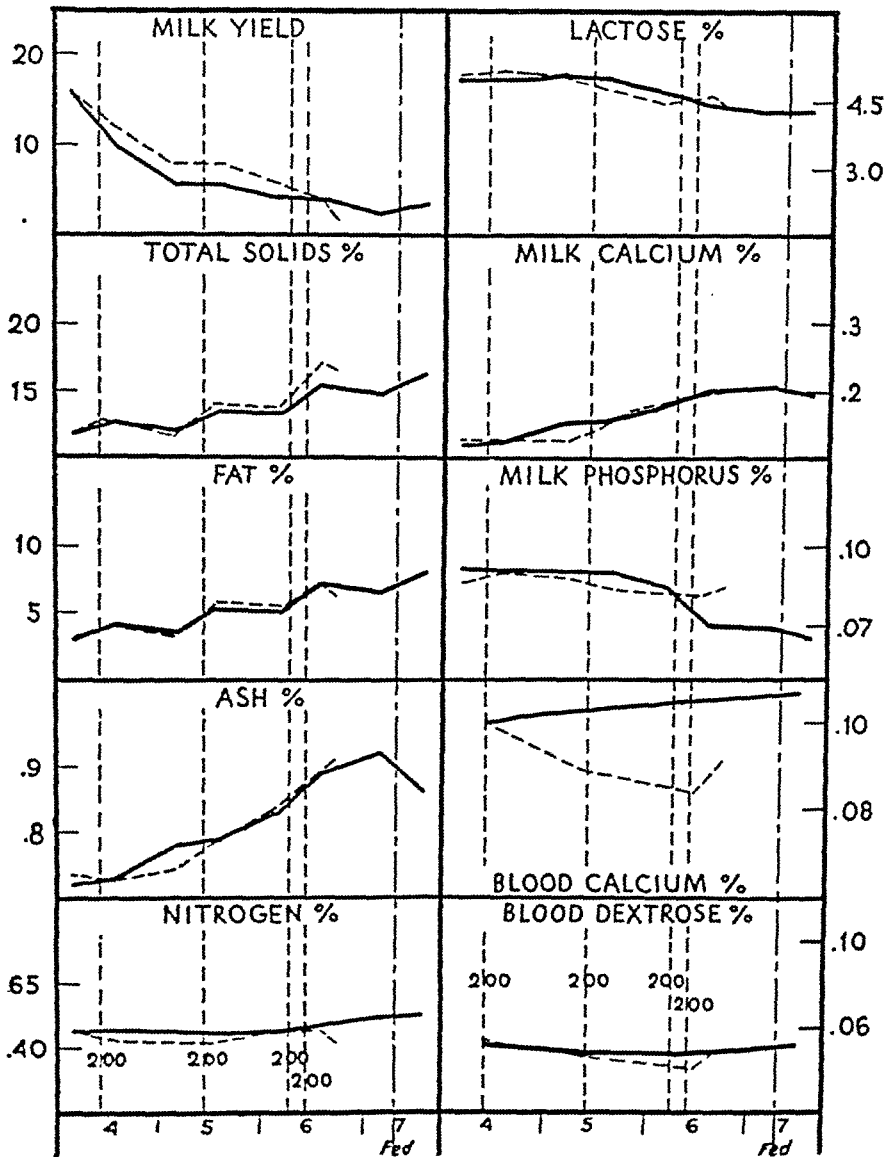


FIG. 9. Cow 47, parathyroid and complete inanition. The vertical dashed lines on the chart show the amount and time that the parathyroid hormone was injected. The solid line represents the data of this experiment,—the dotted line the data for the same cow on inanition alone.

results are, therefore, to be interpreted in the light of starvation experiments rather than as effects produced on the calcium of the blood

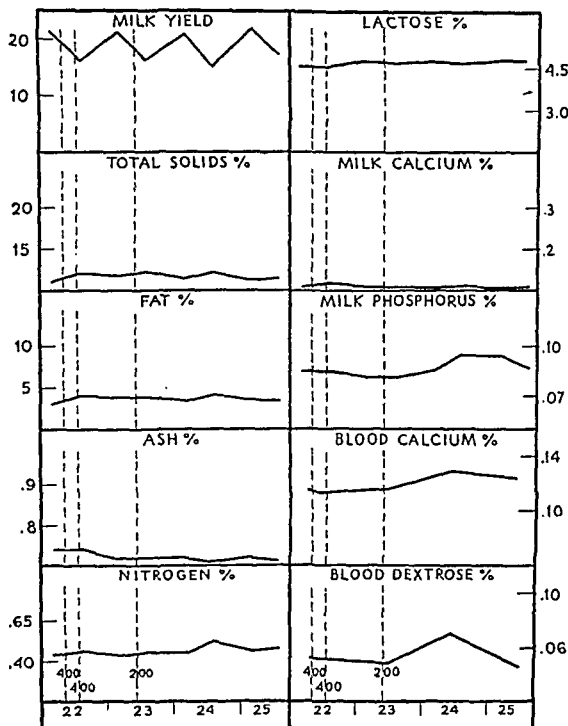


FIG. 10. Cow 52, parathyroid hormone and full ration throughout experiment. The amount of the parathyroid is shown by the vertical dashed lines on the chart at the time when the injections took place.

by the parathyroid hormone. The facts obtained, aside from their own intrinsic interest, are of considerable importance as comparative

STUDIES ON MILK SECRETION

material for the insulin experiments which follow. The charts in Figs. 9, 10, and 11 present this material. Cow 52 received a full

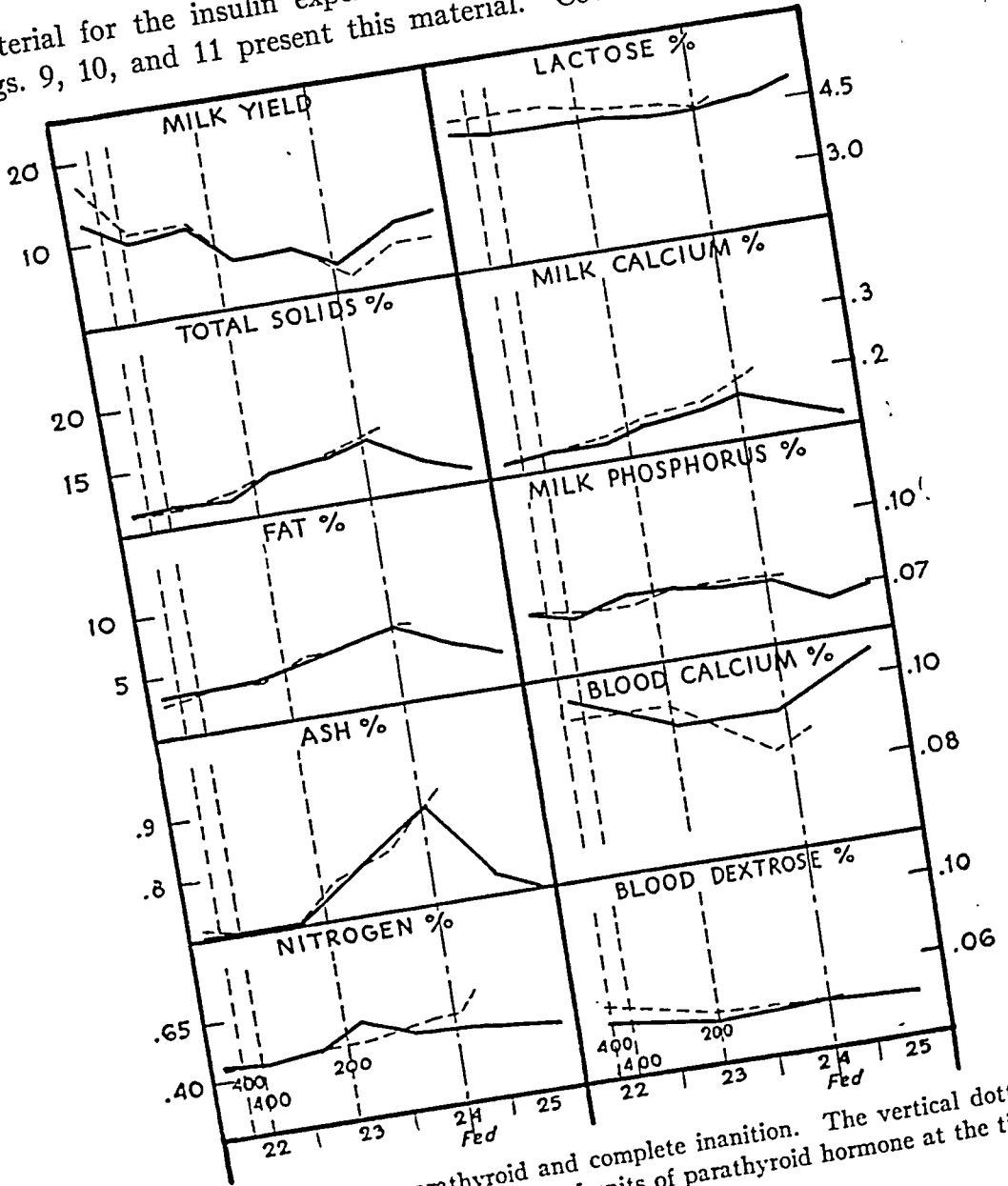


FIG. 11. Cow 212, parathyroid and complete inanition. The vertical dotted lines on the chart show the number of units of parathyroid hormone at the time when they were injected.

ration throughout the experiment. Cows 47 and 212 were under complete inanition. The times and amounts of the parathyroid hor-

mone injected are shown on the vertical dashed lines on the chart. As will be noted in the earlier experiments, these cows were used for previous complete inanition experiments. The results for these earlier experiments are shown on the dotted lines.

Fig. 10 pictures milk secretion by a cow receiving an adequate ration. Attention is called to the relative steadiness in the percentage composition of all of the constituents of the milk, maintaining as they do their normal level throughout the entire experiment. This chart should be contrasted with the other cows under starvation. The graph for Cow 52 contrasts sharply with the one obtained in the starvation experiment. The chart for Cow 52 should also be compared with the subsequent one for Cow 47 when she received insulin and a full ration. The insulin treatment of Cow 47, in contradistinction to the parathyroid treatment of Cow 52 and despite her receiving a full ration, caused all of the constituents of the milk and the quantity of milk produced to show a definite and marked effect of the insulin. It is evident that the results for starvation and insulin are to be accounted for by their effect on milk secretion and not to any extraneous variables.

Figs. 9 and 11, for Cows 47 and 212, show simply the characteristic picture of the effects of starvation on milk production. The milk yields drop; the total solids, butter fat percentage, ash percentage, and calcium in the milk all rise. The nitrogen per cent remains fairly constant. The lactose per cent contrasts with the other elements of milk in that it tends to seek lower levels than are normal. Accompanying this change is a reduction in the dextrose content of the blood during the inanition period. On feeding the animals the different items return to normal.

What, it may be asked, is the mechanism by which these changes are brought about? The most noticeable fact is the difference in the behavior of the lactose and the behavior of the other constituents of the milk. In the case of the lactose the percentage composition decreases while in the case of the other constituents the percentage composition increases. As indicated earlier from the results of the composition of blood before and after it had passed through the udder the conclusion is drawn that the dextrose of the blood is extracted from it as it passes through the udder and is organized into the lactose of the milk by the udder cells. If this conclusion is correct, it would

be expected that if the dextrose of the blood is decreased the lactose of the milk would likewise be decreased if it has no other origin. The fact that this is just what happens in these experiments furnishes further and independent proof for the correctness of the former conclusion; namely, that the lactose of milk is derived from the dextrose of the blood.

This change of the dextrose of the blood and the concordant change in the lactose of the milk is, we believe, a key to the explanation of these results. It has been known for a long time that the milk of individual cows is relatively stable for their lactose, ash, and protein content and less stable for their fat percentage (Eckles and Shaw (13) and others). It is likewise known that under similar normal conditions the constitution of the blood of an animal is relatively stable in its composition for the dextrose, salt, and protein content. The osmotic pressure of the blood and of the milk under normal conditions is essentially the same when the fat, which is not in the same phase as the rest of the system, is disregarded (Jackson and Rothera (14), and Davidson (15)). The osmotic pressures of milk are very largely determined by their lactose content, whereas the osmotic pressures of blood are relatively little determined by their sugar content; the large factor being the salt. It would therefore follow that if the osmotic pressures of the blood and milk are to be kept in constant ratio the milk of a lower lactose content would have to increase its content of salts in a rather pronounced degree to make up for this deficiency of lactose, since the salts are the only other large element capable of contributing to the osmotic pressures of the milk.

The osmotic pressures of the milk may be computed from the millimolar concentration of the lactose and ash found in the milk. A difficulty arises in that the ash is composed of six major osmotically active elements, potassium, sodium, calcium, magnesium, phosphorus, and chlorine, with a little sulfur and iron. The exact analysis of only two of these was obtained, namely calcium and phosphorus. The variation of these two elements indicates that the proportions of all six elements may be assumed as roughly the same as in normal milk ash for the purpose of calculating the osmotic pressure. As pointed out earlier, a decrease in the lactose should materially decrease the osmotic pressure unless the salt content increases to compensate.

Since the latter actually takes place, it is of interest to note the changes in osmotic pressure of milk which result from continued inanition. These are given in Fig. 12.

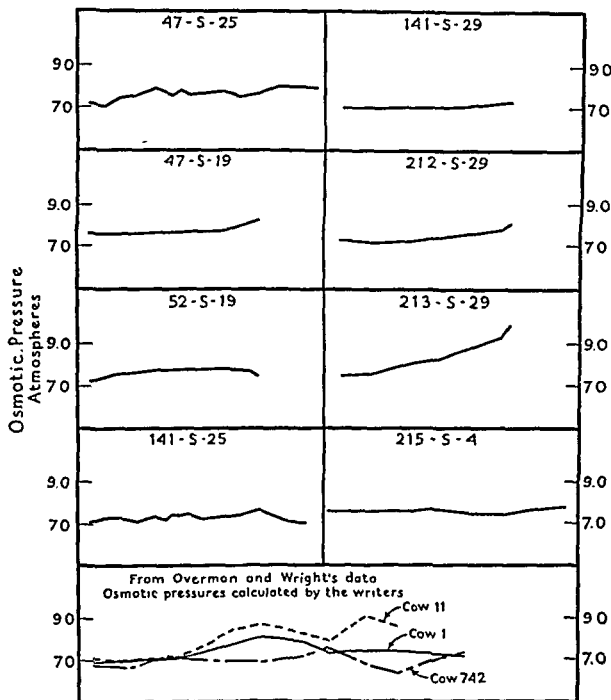


FIG. 12. Relation between the calculated osmotic pressure of milk determined from the proteins, ash, and lactose, in inanition.

Before considering the facts brought out by Fig. 12, it is well to note the changes in the blood during starvation. Mayer (16) has shown that the osmotic pressure of the blood of fasting dogs increases with

the progress of inanition. Tria (17), working with both dogs and rabbits, obtained like results using the method of depressed freezing point. Polányi's (18) experiments on dogs lead to the same conclusion. It therefore seems to be rather conclusively proved that there is an increase in osmotic pressure of blood serum during the progress of inanition. As Morgulis (19) points out this change is apparently due to a greater ash content in the relatively higher proportion of the water soluble salt, chiefly sodium chloride.

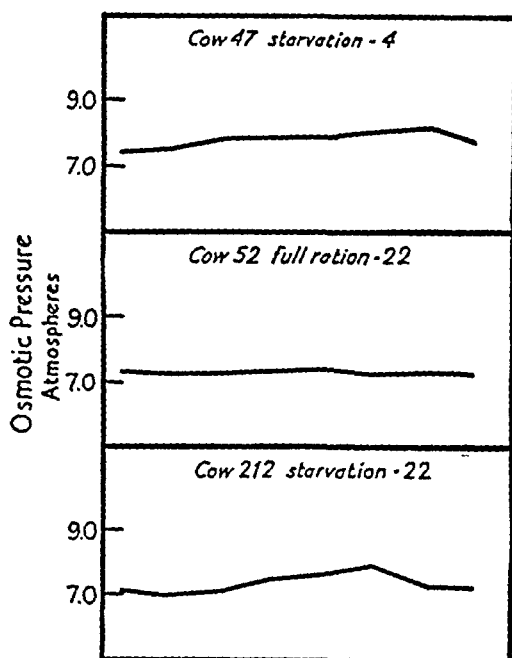


FIG. 13. Calculated osmotic pressures of milks from cows treated with parathyroid hormone.

From Fig. 12 it will be observed that the cows on starvation show a slight general increase in the osmotic pressures of their milk. In only one cow, Cow 213, was this increase marked. This cow is the animal which showed the greatest decrease in lactose in her milk. This decrease was accompanied by the greatest increase in the content of salt and nitrogen. The calcium content of the salt increased 2.4 times while the salts as a whole only increased 1.7 times indicating that the apparent increase in calculated osmotic pressure may be

fictitious for Cow 213 since the calcium salts very possibly combine in the proteins.

If we calculate the osmotic pressures of the milk in the experiment of Overman and Wright, we find that for their three cows they also have an increase in the osmotic pressures of these milks. These changes were most pronounced in cows which were nearing the end of their lactation; whereas for the cow beginning her lactation the osmotic pressure did not rise to any extent.

The osmotic pressures of Cows 47, 52, and 212, treated with parathyroid are shown in Fig. 13. Cow 52 shows that a cow with a normal ration has practically a constant osmotic pressure. The osmotic pressures of the cows under starvation, 47 and 212, rise somewhat toward the last of the experiment, due no doubt to the change in osmotic pressure of the blood during inanition.

The decrease in the lactose of milk results in the necessity of a marked increase in the salt content of the milk if the osmotic pressure of this milk is to remain the same. This increase is actually observed and the osmotic pressures of milk, as determined from its composition, follow those which are to be expected of blood with progressive starvation. We may therefore conclude that the changes in the composition of milk during inanition are the results of the changes in the composition of the blood.

The behavior of the butter fat percentage in contrast to the percentages of the other constituents is of interest in the light of the results of the writers (10) in indicating that the fat of butter fat was first deposited in the udder from the blood and then metabolized into the characteristic fat of milk. The rate of metabolism of this fat is such that there appears to be stored in the udder in advance of milk secretion a limited amount of reserve fat which is equivalent to butter fat. If this is the case, the amount of this fat released into the alveolar spaces of the udder should be somewhat independent of the other constituents of milk. If the proportionate rate of change of the butter fat be examined it is found to increase from $1\frac{1}{2}$ to $2\frac{1}{2}$ times its initial amount as starvation progresses during the experiment. The ash increases to the extent of only 1.1 to 1.3 times the original amount. The nitrogen, on the whole, changes very little. The lactose decreases. These facts indicate that the butter fat is to some

extent independent of the other constituents of the milk. This result is accordingly in agreement with the previous work. It might also be added that the more or less independent movement of the butter fat as contrasted with the other solids is seen in comparing milks of different breeds with differences in fat percentage.

SUMMARY

In this paper data are presented on cows receiving no food but having access to all of the water which they wished. The yield and composition of the milk were determined at various times during the periods of starvation. The composition of the milk showed changes which were progressive in the sense that they followed a definite course. They were characterized by a marked lowering in the amount of milk produced, by an increase in the total solids (chiefly an increase in the percentage of fat and ash, with a slight increase in proteins), and by a pronounced decrease in the lactose. The decrease in lactose corresponded with a decrease in the dextrose content of the blood, thereby supporting the conclusion that the lactose of milk has as its precursor dextrose of the blood. All the changes in milk composition during starvation can be directly related to the simultaneous changes in the blood.

The following companion paper on insulin and phloridzin as they affect milk secretion further develops these hypotheses.

ON THE MECHANISM OF MILK SECRETION

THE INFLUENCE OF INSULIN AND PHLORIDZIN

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Insulin

The introduction of insulin into the blood stream of dairy cattle should markedly lower the blood sugar and bring about the characteristic changes attendant thereto if the hypotheses developed in the previous paper are correct. In the initial experiment of 1924 we were kindly supplied insulin by Armour and Company, through the courtesy of Dr. Frederic Fenger. In all of the subsequent experiments, in fact all of those reported here, we have been supplied insulin by Eli Lilly and Company, through the courtesy of their research director, Dr. George H. Clowes.

The initial experiments were devoted to finding the approximate dose which would affect the blood sugar of cattle. This dose was very much larger than expected; in fact much out of proportion to the size of the animal. From 500 to 1200 units at one time seemed to be the amount necessary to produce the effects desired. The constituents of the blood and the milk were determined in the same manner as indicated in the preceding paper.

The Data

It was first thought by the writers that insulin could produce its effect in extreme form only in the presence of complete inanition. For that reason the first three cows treated were given only water. For the fourth cow, No. 47, no change whatever was made in her diet; this cow receiving her ordinary ration plus all the water that she wished.

The milk yield, the composition of the milk, and blood are all plotted against time and appear in Figs. 1, 2, 3, and 4. The units of

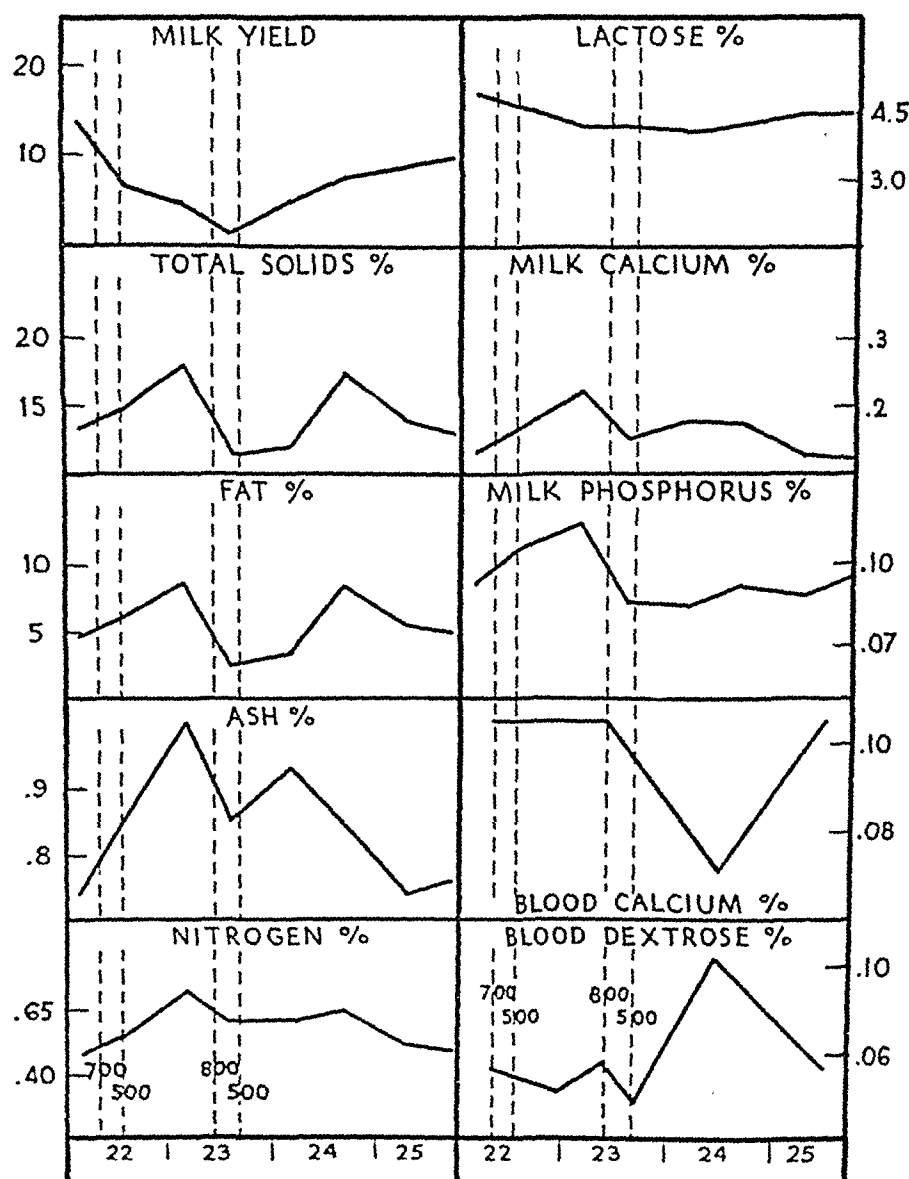


FIG. 1. Cow 47, the effect of insulin on milk secretion. Insulin treatment with full ration throughout is represented by the solid line.

insulin introduced into the blood are indicated by the dotted lines on the chart. The position of the vertical line represents the time at which the insulin was introduced into the jugular vein.

The immediate effect due to the introduction of insulin into the blood stream of the cow is to cause a sharp drop in milk production. This drop is more pronounced than is the drop for starvation. It

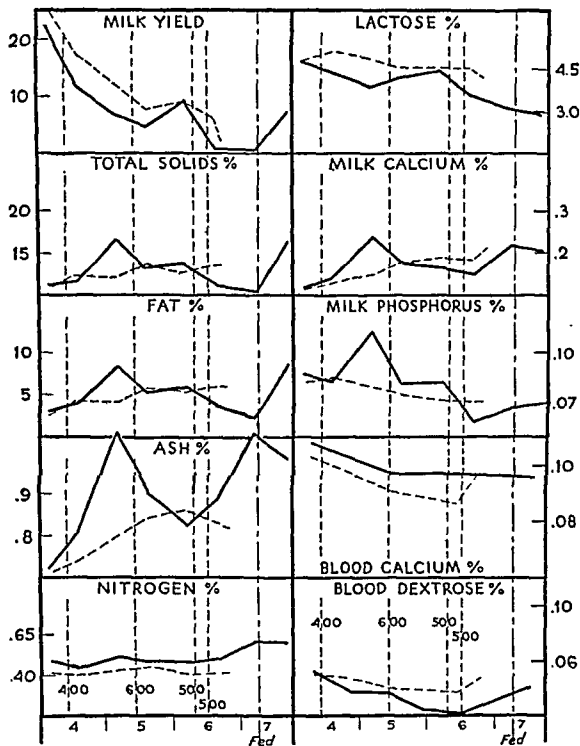


FIG. 2. Cow 52, the effect of insulin on milk secretion. Insulin with starvation is represented by the solid line; the dotted line shows the same cow with starvation only.

MECHANISM OF MILK SECRETION

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occurs whether the cow is kept under starvation or allowed her normal ration. The effect of the insulin treatment were off much more rapidly when Cow 47 was fed during treatment than when on starvation. The effect of the insulin on Cow 141 in her second period was much more

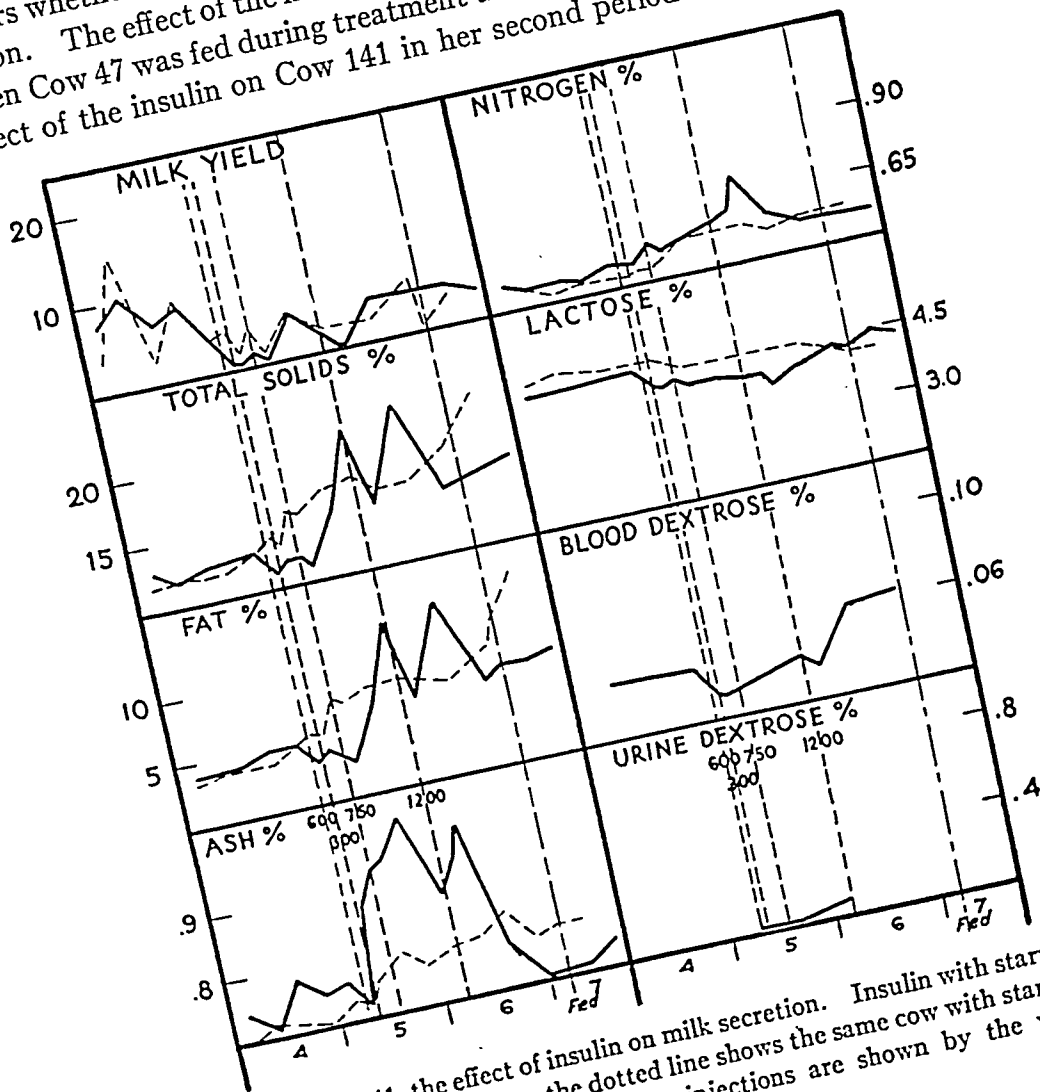


FIG. 3. Cow 141, the effect of insulin on milk secretion. Insulin with starvation is represented by the solid line; the dotted line shows the same cow with starvation only. The amount and time of insulin injections are shown by the vertical dashed line.

severe than it was for the other animals, as noted by the fact that she remained in coma for nearly 48 hours even though fed large quantities of dextrose and cane sugar. This severe reaction influenced the rapidity of her recovery in a marked degree, as noted by the length of time it

took her to return to normal production and the changes observed in the composition of her milk during this period.

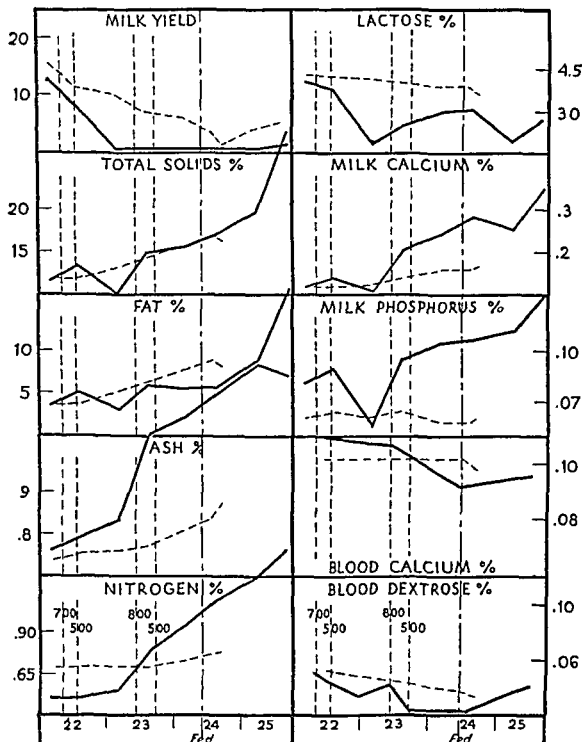


FIG. 4. Cow 141, the effect of insulin on milk secretion. Insulin with starvation is represented by the solid line; the dotted line shows the same cow with starvation only.

The total solids of the milk rose sharply after the introduction of insulin. The highest point reached by this rise in total solids was much greater than that noted in starvation for the same period; in fact the effects 16 hours after the introduction of insulin correspond to those found where the starvation has endured for 48 hours. From this high point the total solids tend to decline; the decline being most rapid in the cow which was fed. With the subsequent injection of insulin the total solids rose somewhat but not to the same extent as noted in the first instance. Cow 141, on the second experiment where the effect was so long protracted, showed a tremendous but late rise in her milk total solids, which was maintained for a period of 2 or 3 days, when it declined. This late rise was apparently due to the insulin shock through which Cow 141 had passed and the attendant rather protracted inanition.

The changes in the butter fat percentages were equally acute. A rise in the butter fat percentage to a level which was normally reached by starvation in 2 days was reached by the cows on insulin treatment in 16 hours. From this point the butter fat percentage declined on the third injection of the insulin. The curves of the butter fat percentage are quite different in the insulin treated cows than those for starvation alone.

The percentage of ash rose very rapidly after the introduction of insulin. This rise was noted whether the cows were starved or fed. The point reached by the rise in 16 hours was as high as that found for starvation after a period of 48 hours. The height of the rise was somewhat affected by whether or not the cow was fed; the cow being fed not going as high as the cow which was under starvation. The high point of the ash percentage on the first rise was about 10 per cent above normal. On the subsequent introduction of the insulin a second rise took place although not to the same level as the first one.

The nitrogen content of the milk behaved irregularly. Cow 52 under complete starvation had a slight rise in the amount of nitrogen with the introduction of insulin. This rise was probably not significant. Cow 47, adequately fed, showed a marked rise in the nitrogen value, about 1.5 times normal, from which point it declined somewhat to reach normal at the end of 3 days. Cow 141 in the first experiment showed a slight rise in nitrogen per cent at first and a marked rise

after the third injection of insulin. Cow 141 on the second experiment showed little change in the nitrogen content of her milk after the first two injections, but after the third the rise was marked and continued. This cow's whole chart is of sufficient interest to warrant a separate chart to show the full trend of the cow's production on a smaller scale (Fig. 5).

The changes in the lactose per cent were equally striking, but in the opposite direction from those above noted. The lactose dropped rapidly to values which were a tenth lower than those noted under starvation. From these values the lactose tended to return somewhat toward normal until a subsequent injection of insulin when the drop was continued. The drop for Cow 141 in the second experiment was very severe indeed going to a lactose value less than half that normally found. Cow 52 showed a very pronounced drop when the second group of insulin injections was made.

The percentages of calcium and phosphorus in the ash of the milk tended to follow those of the ash itself, indicating that the ash composition was probably not changed markedly but was simply increased in amount.

The blood sugar percentages furnished some measure of the insulin effect and also of the amount of dextrose which would be available to the udder for the organization of the lactose of milk. The introduction of insulin into the blood stream has the very rapid effect of lowering the blood sugar. These blood sugar values sometimes go to the very low value of 20 mg. per 100 cc. or less. For the amounts of insulin used it would seem that the period of time necessary to accomplish this result was more than half a day. On the introduction of a subsequent injection of insulin the blood sugar tends to fall again sometimes to a new low level. From this point, if the cow receives no further interference, she slowly builds up the blood sugar to a point which, if she is receiving an adequate ration, may go considerably above the normal.

Before discussing these results, it is of interest to look at the curve for Cow 141 on the second experiment over a longer period of time. This cow was given the largest injections of insulin and over a shorter time, plus starvation during the period. She was much more markedly affected by the insulin than was Cow 52 which received insulin

over a longer period of time, and to only 80 per cent of the amount. Shortly after the third injection of 800 units of insulin Cow 141 became

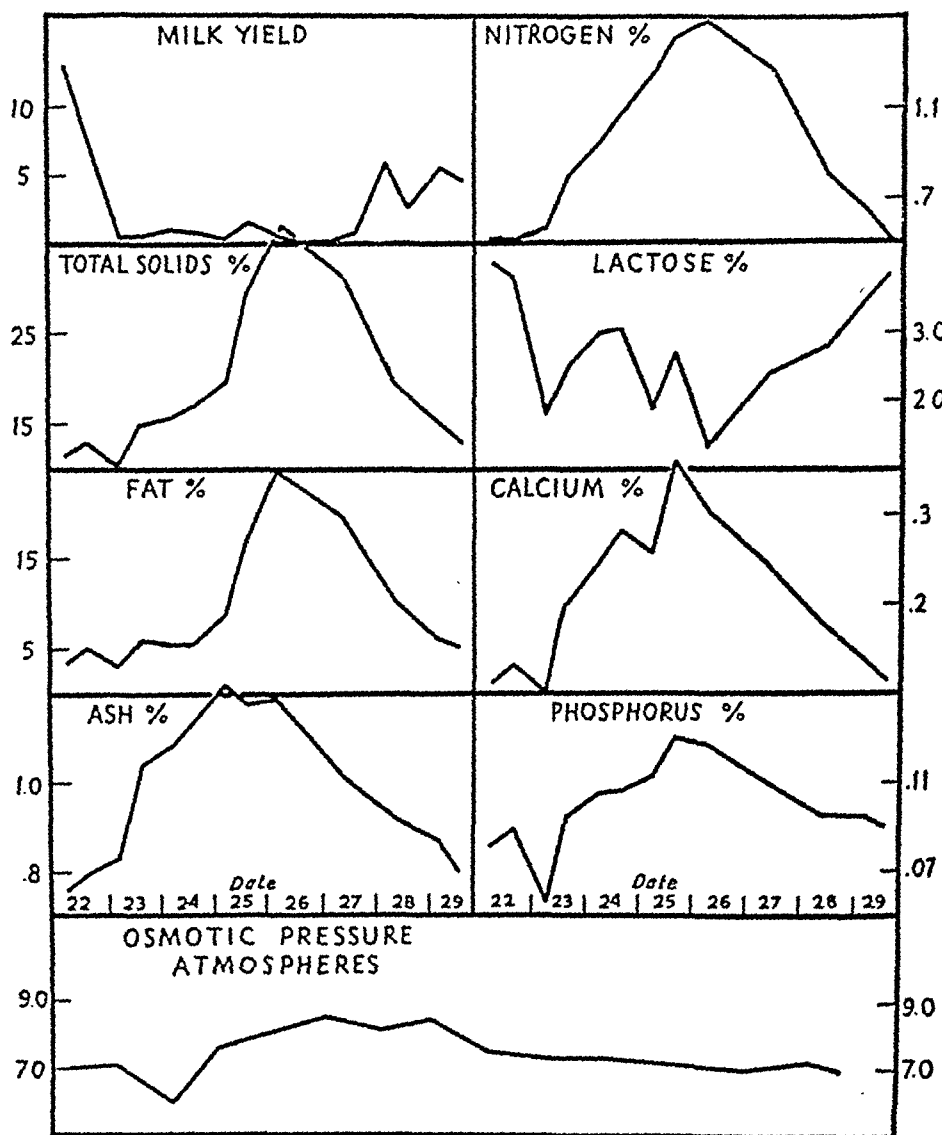


FIG. 5. Milk yield and composition of the milk of Cow 141 during insulin treatment and recovery.

partially paralyzed. After the fourth injection of 500 units the cow could no longer stand or use her legs but could only lie on her side. Her breathing was irregular; the limbs became cold. The cow then

passed into coma and remained in this condition for about 12 hours when she was given an injection of 20 cc. of 10 per cent dextrose solution directly into her jugular vein. At the same time she was fed 500 gm. of dextrose as a water solution. In 20 minutes or so the cow was on her feet. But a short time after that she returned to her lying position and in spite of giving her further injections of dextrose and of feeding her large quantities of cane sugar no subsequent improvement took place for another 26 hours; a gradual improvement then commenced. At the end of about a week the cow's milk production returned to 75 per cent of her normal production and she was in other respects apparently well. The clinical aspects of this case clearly indicate that this cow was much more markedly affected than she was on her previous treatment the year before, or than was Cow 52 treated during the same time. This cow is, so far as the writers are aware, the only one exhibiting artificially produced insulin shock over a protracted period. The ability to produce such a prolonged shock, especially after the feeding and injection of dextrose, is therefore of much interest in view of the suggestion that milk fever is due to such a hypoglycemia of the blood brought on by a heavy milk secretion. The course of this cow's milk production and the composition of the milk in these periods are of significance to the study in hand. Fig. 5 gives the extended graph for this cow during the whole period but on a reduced scale.

The curve of milk production for Cow 141 over this extended period is characterized by a rapid drop in the quantity of milk produced after 16 hours from the time the first insulin was injected. This low production is continued over a period of 4 days, a length of time much longer than for the other cows which did not receive as severe a shock from the insulin. This is no doubt partly to be accounted for by the starvation attending the coma after the last injections of insulin. It is, however, to the insulin which must be attributed the majority of the effects since this cow was fed large quantities of sugar during this coma period. It is of interest to note that in spite of the severe effect of the insulin this cow returned to normal production after a time. The period is, however, much longer than for the cow fed during the insulin treatment, or for a cow given less insulin although starved during the period.

The highest total solids content of Cow 141's milk is reached at the relatively late period of 60 hours after the last insulin injection. The total solids content goes to a very high level resulting in a milk which is nearly solid. These levels are about three and one-half times the normal; the total solids content finally reaching 37 per cent. This rise is quite largely accounted for by the increase in the butter fat content of the milk from about 3.7 per cent to 24.8 per cent, or to 6.7 times the normal value.

The ash content likewise increases from 0.74 to 1.22 per cent, an increase of roughly 60 per cent. The curve for percentage of ash, while it rises, does not exactly parallel that for the butter fat percentage, as it reaches its crest at an earlier period and remains fairly constant at this level for a much longer time.

The nitrogen content of the milk likewise increases to a relatively high level. This increase is of little significance until the third injection of insulin when the rise commences. The high point is reached at a later period than that for the ash but at an earlier period than that for the butter fat percentage. The amount of nitrogen contained in the milk at that period is about three times as much as that ordinarily found. From this high point the nitrogen drops off and by the end of the experiment has very nearly reached normal.

The lactose percentages behaved irregularly but are throughout inverse to those of the other constituents of the milk. Within 16 hours after the injection of the first insulin the lactose of the milk has reached very low values. From this low point of 1.8 per cent there is a slight recovery until the effect of the second insulin injection is noted when the lactose returns to its low values. The lactose keeps these low values as long as the ash percentages remain high but when the ash percentages start to drop the lactose returns to normal. At the end of the experiment it is within 0.6 per cent of the normal value for this cow.

The calcium content of the ash follows the changes of the ash in large measure. It does, however, reach relatively higher values being at the crest of the curve about three times its normal value, whereas for the ash it is only 1.6 times the normal value. This possibly may be accounted for by the protein increase and the affinity of casein for calcium. The calcium content has returned to nearly normal values

by the end of the experiment. The high point of the calcium corresponds exactly with the high point of the nitrogen.

The phosphorus content of the milk parallels almost exactly the calcium of the milk. Unfortunately the blood sugar and calcium were not followed further than the period noted on the earlier chart.

These studies show that the effects of insulin on milk secretion are much more severe than those of starvation in that the response is more immediate. They are characterized by a rapid drop in milk production, an increase in the total solids, butter fat percentage, ash, and to a limited degree in nitrogen. The changes in ash content appear to be fairly uniform for its constituents as the calcium and phosphorus very largely parallel the changes in the total ash. Lactose contrasts with the other milk constituents in that it rapidly declines in amount. It is also to be noted that the butter fat percentage does not appear to take the same course as that for the starvation results. The increase for insulin being almost immediate and then held at that level rather than a continuous increase over the period. Insulin results in a marked decrease in the dextrose content of the blood. The fact that this decrease in dextrose content is accompanied by a marked decrease in the lactose content of milk furnishes another independent link in the proof that lactose of milk comes from dextrose of blood.

As indicated for the starvation experiments, if the osmotic pressure of the milk is to remain constant in view of the decrease in lactose the salt content of this milk must increase rapidly. This is what actually happened. It is of interest to see how nearly alike the osmotic pressures of different periods in the experiment may be. Fig. 6 shows the graph for the osmotic pressures of the milk for the duration of the experiment.

Cow 47 on insulin treatment and full ration showed a slight increase in osmotic pressure for the third injection of insulin. The osmotic pressure after this point, however, returned below normal and remained essentially normal during the entire experiment.

Cow 52 on insulin treatment and starvation showed a like increase in osmotic pressure by the third injection. From this point, however, the milk returned to normal osmotic pressure and remained so throughout the experiment.

For Cow 141, despite the extreme variation which was produced in

her milk, the osmotic pressure of this milk increased only a small relative amount; the increase being at maximum about $1/5$ above normal.

In view of the fact that starvation increases the blood's osmotic pressure to some degree, the increase in the milk's osmotic pressure was to be expected rather than otherwise. The results of the experi-

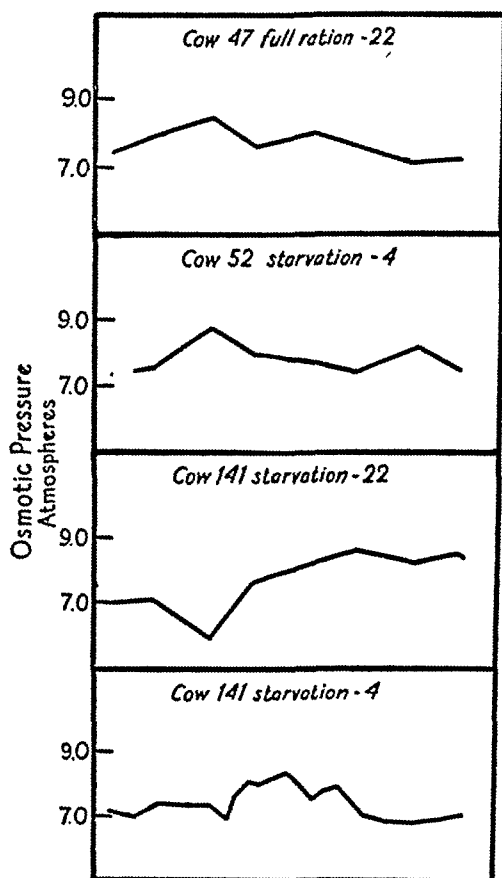


FIG. 6. Calculated osmotic pressures of cow's milk during insulin treatment.

ments on insulin are in agreement with those on inanition in showing that the osmotic pressure of the blood is a leading factor in stabilizing the concentration of the solids found in milk.

If the trend of the butter fat percentages be compared with the trend of the other constituents in the milk, it will be noted that, as in the case of the starvation experiments, the percentages of butter fat

are throughout somewhat independent of the other constituents. The results are consequently in accord with what would be expected from the previous work.

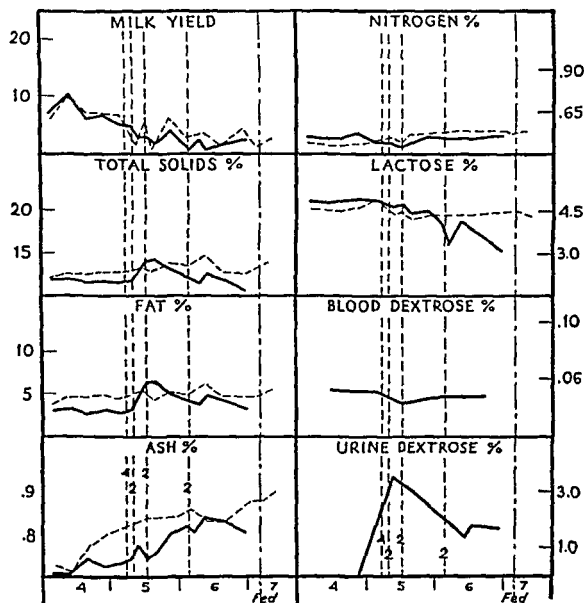


FIG. 7. Cow 47, phloridzin and complete inanition. Vertical lines on the chart show the amount and time of phloridzin injection. The solid line gives these results,—the dotted line gives the data on starvation alone.

Phloridzin

Since phloridzin has the property of causing the animal to excrete large quantities of sugar and nitrogen in the urine, and as a consequence to deplete the blood sugar after a time, it would seem as if its action should result in raising the ash content of milk and in decreas-

sugar has been depleted. This result is different from that observed in the insulin experiment and as noted earlier is to be expected from the difference in action of the drugs.

The tests on the blood sugar show that phloridzin depleted the blood sugar to a limited degree but that the cow recovered her normal blood sugar in a relatively short period after the introduction of the phloridzin. The degree of depletion through the action of phloridzin is not as pronounced as that noted for insulin.

The urine nitrogen and the urine dextrose were markedly increased by the introduction of the phloridzin as has been noted in all corresponding experiments.

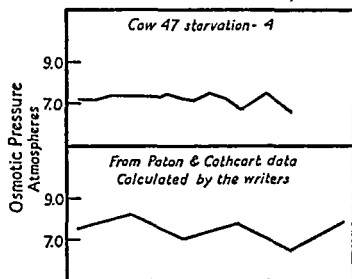


FIG. 8. Calculated osmotic pressures of cow's and goat's milk when under phloridzin treatment.

So far as milk secretion is concerned the results are an increase in the fat and ash content of the milk, a decrease in the lactose percentage and a decrease in the total milk flow. The depressed dextrose of the blood without the corresponding changes in the blood salts, save for a slight depression of the potassium in phloridzin diabetes, will account for the observed results in the milk secreted.

The approximate calculated osmotic pressure of this milk and of the goat's milk in the experiments of Paton and Cathcart are presented in Fig. 8. These osmotic pressures are found to be constant throughout the duration of the experiment indicating again the balanced condition between the salts and sugars of blood and milk in milk secretion.

DISCUSSION AND SUMMARY

The four types of experiments on milk secretion herein described really fall into one general class so far as the physiological effects produced are concerned. Starvation lowers the blood sugar and raises the osmotic pressure of the blood. The experiment using parathyroid hormone with or without starvation may have its effects interpreted as simply due to starvation since 1000 units of this hormone produced no visible effects on the blood calcium or milk constituents different from those of starvation. Since insulin produces a marked and rapid drop in blood sugar it too may be looked upon as a rapid starvation effect. It has some other important effects, however. Briggs *et al.* (21) have shown that potassium and phosphorus of the blood are decreased and Luck, Morrison, and Wilbur (22) indicate a reduction in the amino acids of the blood in insulin treatment. Phloridzin lowers the threshold for sugar retention with the consequence that in time it tends to lower the sugar of the blood to an even greater extent than that noted in starvation. It tends to depress the potassium, to increase the phosphorus content of the blood, and to cause the body to burn protein rather than carbohydrate, thus increasing nitrogen excretion.

All of the experiments are characterized by a sharp reduction in the milk yield. Cary and Meigs (23) have studied like reductions in milk yield produced by varying the energy or protein of the diet. They conclude that such decrease in milk production may be interpreted as due to the direct effect of the starvation and the consequent reduction of the energy and protein available to milk secretion.

The reduction in milk yield for the experiments herein described can undoubtedly be attributed to the same causes as those cited by Cary and Meigs. The experiment where Cow 47 was given a full ration and at the same time injected with large quantities of insulin is of particular interest in this connection. The ration was adequate and the cow ate well, yet her production declined to a fifth of her normal milk yield. Her chart shows that there was a slight reduction in her blood sugar when insulin was introduced into the blood stream. It seems furthermore likely that this sugar was not as available to milk secretion, since there appears to be more than a corresponding drop in the lactose content of the milk. The work of Luck *et al.* would

seem to indicate that there should be a like drop in the amino acids of the blood. These two conditions would lead, according to the work of Cary and Meigs, to a reduction in the concentration of the nitrogen of the milk. Actually, in the experiment as it was performed, the nitrogen increased to a value about 40 per cent above normal. A somewhat similar conflict is noted in two of the other three insulin experiments where starvation accompanied insulin injection. To this extent it would seem that the factor deserving most emphasis in its immediate effect on milk yield is the energy available, and that the later and more secondary factor is the amino acid concentration of the blood.

In the starvation experiments, the butter fat percentage of the milk rises rather uniformly with the duration of starvation. In the insulin experiments, however, the charts appear to show a marked reduction in this butter fat percentage immediately after the introduction of insulin. This is particularly noticed after the second and third injections. Since the dextrose of the blood tends to be reduced and made unavailable to the general physiological processes by the presence of the large excess of insulin, and since this reduction of the butter fat percentage is noted as an accompanying phenomenon, it would appear that the blood dextrose plays a part in the synthesis of milk fat as well as being the source of the milk lactose, possibly as a source of energy in converting body fat to butter fat. In this regard the results for the treatment of Cow 47 with phloridzin are of importance. As noted by others, the introduction of phloridzin causes a marked rise in the fat percentage of the milk. The lactose per cent is also higher than that noted in starvation. Since phloridzin, by lowering the threshold for the blood sugar, causes large quantities of it to be drained from the body through the urine, and therefore reduces the reserve supply, it follows that if the insulin hypotheses are correct we should expect an eventual lowering of the lactose and of the fat below the starvation level. During the last of the experiment this is what was actually observed.

The effects of starvation and of insulin furnish concordant proof for the theory that the lactose of milk is derived from the sugar of the blood.

The fact that the different constituents of the milk, the fat, the

lactose, the nitrogen, and the ash, do not exactly parallel each other in their behavior throughout these experiments indicates that they have in all probability separate origin. This is particularly true of the butter fat percentage, which appears to have a rate of secretion which is more or less independent of the other constituents, and higher in amount. This result would fall in line with the conclusion of the writers in a previous paper in which it was indicated that the fat of the blood was very likely deposited in the udder as fat corresponding to body fat from which source it was metabolized into the fat of milk shortly before it was needed for milk secretion.

The wide variation brought about in the constituents of the milk by the treatment all point to the conclusion that in milk secretion a balance is maintained between the osmotic pressure of the milk and of the blood. Thus when the sugar of the milk is reduced either through starvation or by insulin the ash constituents rise to compensate for this reduction and make the osmotic pressure of the milk similar to that of the blood. These results further appear to indicate that the salts and the sugars are more or less independent in their passage and metabolism into milk from the other constituents. These observations are therefore in line with those obtained by Jackson and Rothera (14) and by Davidson (15) in their brilliant experiments where they modified milk secretion by returning milk or milk sugars and salts to the udder.

These experiments give direct proof for the conclusion that modifications of the blood of dairy cattle produce direct and predictable modification of the milk secreted.

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TEMPERATURE CHARACTERISTICS FOR THE PRODUCTION OF CO₂ BY GERMINATING SEEDS OF LUPINUS ALBUS AND ZEA MAYS

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I

Temperature characteristics for the consumption of oxygen by germinating seeds of *Lupinus albus* and *Zea mays* have been discussed in a previous paper (Tang, 1930-31). The present account deals with the production of CO₂ by these seeds as a function of temperature. It was hoped that such a study might contribute to knowledge of the mechanism of the two phases of respiration, and also to a qualitative investigation of the "respiratory quotient" as a function of temperature.

II

It is of course desirable to use single seeds, as was done for the measurement of oxygen consumption. Several attempts to use single seeds or a very small number of seeds (six to twelve) failed to give significant results, owing to the small amount of CO₂ produced (*cf.* Navez, 1928-29, and Crozier and Navez, 1930-31), and finally a mass method was adopted. Thirty seeds in the case of *Lupinus*, and fifty in the case of *Zea*, were used for each test. The usual Ba(OH)₂ absorption technic was used for collecting the CO₂ excreted. A stream of outside air freed of dust particles by passing through about 20 cm. of loosely packed cotton wool was sucked through metal piping. The CO₂ in the air was eliminated by passing it through about 120 cm. of soda-lime, and then through three bottles of concentrated NaOH solution. The CO₂-free air was saturated with water vapor by passing through a moist chamber containing distilled water and glass beads, and was then led into the respiration chamber. The air, together with the CO₂ excreted by the seeds, was then allowed to pass out of the chamber and bubbled through a special Pettenkofer tube (*cf.* Crozier and Navez, 1930-31) containing 50 cc. of about 0.383 N Ba(OH)₂, where the CO₂ was completely absorbed. The suction was by a water pump and the rate of air passage was about 250 cc. per minute, maintained constant by means of a Hoffman clamp between the respiration chamber and the inlet to the Pettenkofer tube.

The respiration chamber was made of a piece of straight Pyrex glass tubing 32.5 cm. long and 2.5 cm. in diameter, with three outlets for the insertion of thermocouples (*cf.* Crozier and Navez, 1930-31). The chamber was closed at the ends by one-holed rubber stoppers through which the inlet and the outlet tubes passed.

The seeds were placed on a piece of wire netting (about 5 mm. by 5 mm. mesh) about 26 cm. long and 2.3 cm. wide, of which the wires were thoroughly covered with paraffin. The seeds were so placed on the netting that they were isolated from one another and their hypocotyls or radicles were free from contact and gravitational stimuli. The netting, with the seeds on it, was then carefully inserted in the chamber which was held horizontally in the thermostat.

The Ba(OH)₂ solution was prepared according to Kostychev (1927). 7 gm. of crystalline Ba(OH)₂ was dissolved in each liter of water to which was added 1 gm. of BaCl₂. The concentration of such a solution was about 0.383 N, varying slightly each time it was prepared. The actual concentration was ascertained by titrating against the HCl solution which in turn was titrated against Na₂CO₃ solution. The HCl solution was prepared by diluting concentrated acid (C.P.) to about 5/100 N and titrated against Na₂CO₃ solution of known strength, using phenolphthalein as indicator. When so prepared, each cc. of HCl solution was found to be equivalent to 0.845 mg. CO₂.

The amount of CO₂ excreted by the seeds was ascertained in the following manner. The Ba(OH)₂ solution from the Pettenkofer tube was run into an Erlenmeyer flask and tightly stoppered, and let stand for an hour for the carbonate to settle. A sample of 10 cc. was then pipetted out into a second Erlenmeyer flask (150 cc. capacity) and two drops of 1 per cent phenolphthalein solution added. HCl from a 25 cc. burette graduated to 5/100 cc. was run into the flask with constant shaking of the latter until neutrality was reached. The end point was observed under a daylight lamp. The titrations were done in triplicate, and they seldom showed a variation of one part in a thousand. At the beginning, and sometimes also at the end of an experiment, a blank titration was performed in which no seed was placed in the chamber. The volume of HCl used was recorded, and from this quantity was subtracted the volume of the solution used in each actual experiment. The difference, multiplied by the "CO₂ equivalent" (5×0.845) gave the mg. of CO₂ produced in a given period by the group of seeds.

The thermostat used is of the type described by Crozier and Stier (1926-27) and is capable of maintaining a temperature constant to $\pm 0.005^\circ$ between 0 and 50°C. For each kind of seed a test was usually started at 18°; the temperature was then lowered to 14°, and then to 12.5°. After that the temperature was successively raised to 16°, 20°, 22°, and 24°. This was then repeated in another experiment at the same temperatures or at different temperatures, so that the resulting data, when brought together, gave rates of CO₂ production between 12.5° and 25° about 1° apart. The values of the temperature characteristics for the individual experiments were found to be identical.

1 hour was allowed for thermal adaptation, during which time the air stream was allowed to proceed. At the end of the period of adaptation, the stream was

temporarily cut off by proper turning of the stop-cocks on the Pettenkofer tube, and 50 cc. of $\text{Ba}(\text{OH})_2$ solution was introduced from an 8 liter reservoir placed above and connected with the tube. The passage of the air was then resumed and the CO_2 absorbed by the baryta water. At the end of an hour the air stream was again stopped for about 2 minutes to allow the collection of the baryta water for titration, after which the streaming was again resumed. At the same time the temperature of the thermostat was changed, and the seeds were allowed to adapt for another hour at the end of which the entire procedure was repeated. The experiments were carried out in darkness except during the time of collection and changing of the temperature of the thermostat when the light was turned on for a few minutes, the thermostat being covered with red cloth and boards to prevent the light from reaching the respiration chamber.

The seeds used in this study belong to the same lots as those used in the measurements of the rate of oxygen consumption, and were germinated in the same manner, i.e., on moist maple sawdust, in darkness, at $23 \pm 1^\circ\text{C}$. Seeds were soaked in distilled water for 12 hours, then incubated on sawdust for 12 hours (*Lupinus*) or 36 hours (*Zea*).

III

Fig. 1 presents the data for *Lupinus albus*. The ordinate is logarithm of rate of CO_2 production, the abscissa the reciprocal of absolute temperature. The values from several experiments were brought together by factors. Within reasonable limits (± 8 per cent at the maximum) the points fall well on two lines intersecting at a critical temperature of 20° . The temperature characteristic (μ) for the line above the critical temperature is $16,100 \pm$ calories and that below, $24,000 \pm$ calories.

The results of the experiments with *Zea mays* are given in Fig. 2, plotted in the same manner as Fig. 1. There is no evidence of any discontinuity of the line on which the points fall. The deviation from the straight line of the two points at the lowest temperatures is mainly due to the difficulty in estimating the small amounts of CO_2 at those temperatures. But for these two exceptions, all points fall well on a line from the slope of which $\mu = 20,750$.

Plotted in a slightly different way, we have Figs. 3 and 4 corresponding to Figs. 1 and 2 respectively. The relative rates of CO_2 production in mg. per hour per 30 or per 50 seeds are plotted on the ordinate and the temperature in degrees C. on the abscissa. For *Zea*, the curve so obtained is of course continuous; while for *Lupinus* there is no way of fitting the data but by two curves—each exponential

CO₂ PRODUCTION OF GERMINATING SEEDS

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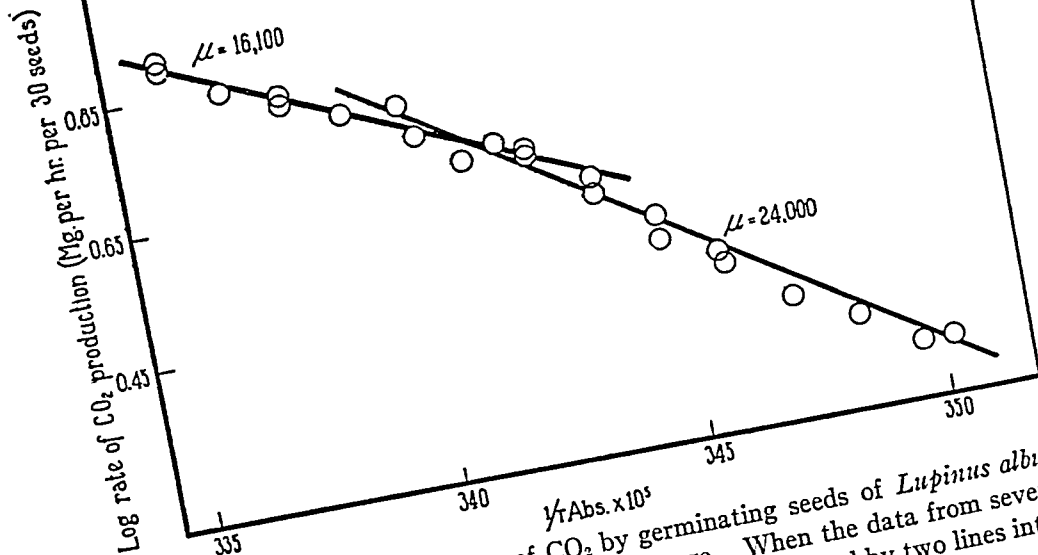


FIG. 1. Log rate of production of CO₂ by germinating seeds of *Lupinus albus*, plotted against reciprocal of absolute temperature. When the data from several experiments are brought together by factors, they can be fitted by two lines intersecting at 20°, with a maximum scatter of about ± 8 per cent.

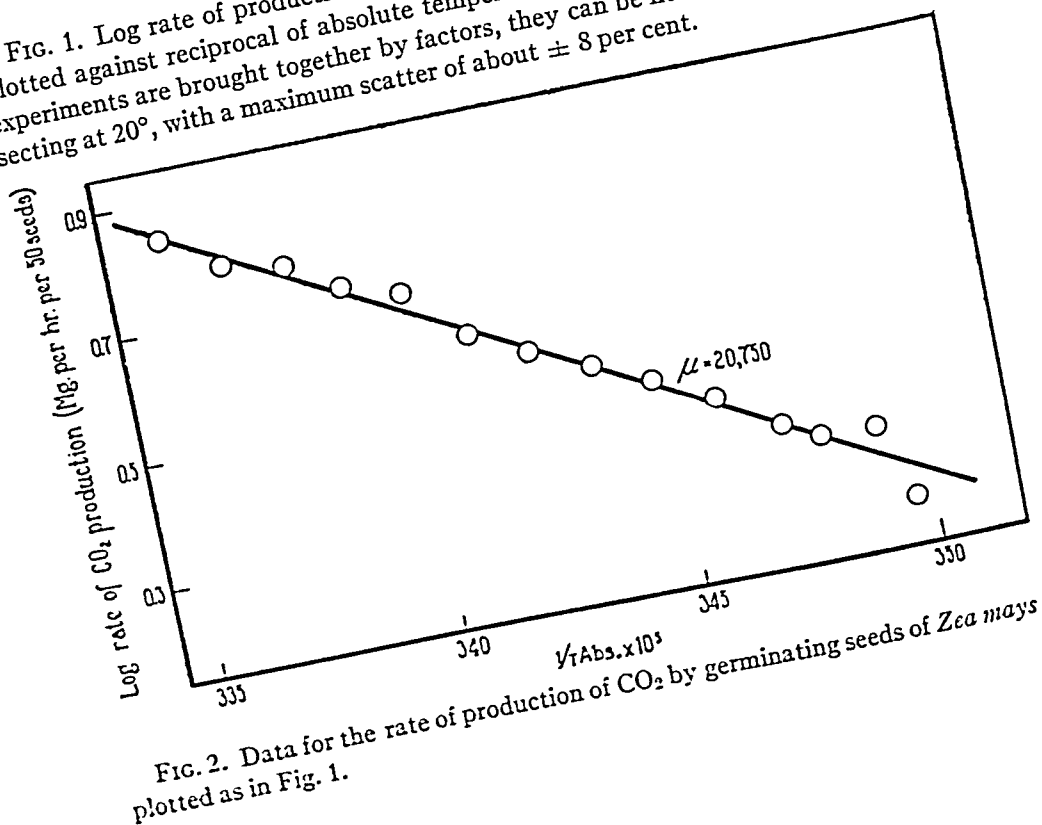


FIG. 2. Data for the rate of production of CO₂ by germinating seeds of *Zea mays* plotted as in Fig. 1.

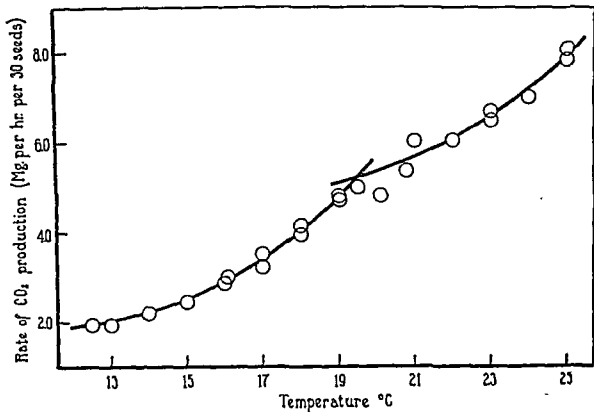


FIG. 3. Data from Fig. 1, plotted in a different way. The ordinate represents relative rate of production of CO₂ in mg. per hour per 30 seeds, the abscissa temperature in degrees C. The cusp formed by the two intersecting lines (from Fig. 1) brings out the occurrence of the critical temperature. The points tend to scatter with increasing temperature—especially so at the region of the critical temperature (*cf.* Crozier and Stier, 1926–27).

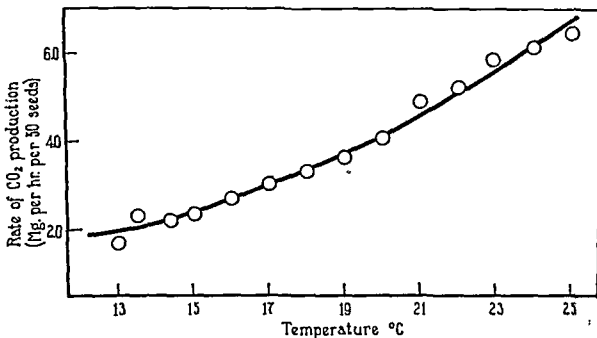


FIG. 4. Data from Fig. 2 plotted as in Fig. 3. The points can be fitted by only one curve, with no evidence of a critical temperature at 20°. The points tend to scatter more as temperature increases.

in nature, intersecting at 20° and forming a cusp at that point. This fact is interesting not only because it illustrates the necessity for the discontinuous graph in Fig. 1 (*cf.* Crozier, 1924-25; Brown, 1926-27) but also because it brings out the fact that in this type of experiment, it is essential to determine the rates at short intervals of temperature instead of as a few scattered points, as has been done by many workers (*e. g.* Warburg, cited by Harvey, 1930, p. 327; *cf.* Crozier and Stier, 1926-27). Thus if we obtained only the points at 13°, 16°, and 24°, we could have drawn a smooth curve through these three points, completely failing to detect the "break" at 20° in the case of the *Lupinus* seeds.

IV

It is unfortunate that most of the recent data dealing with production of CO₂ by seedlings as a function of temperature have not been obtained in a manner warranting the mode of treatment used here. Experiments with wheat seedlings reported by Mack (1930) are complete and well planned in many ways, but the failure to eliminate geotropic and contact stimuli renders the data unfit for quantitative treatment. Those reported by Kurbatov and Leonov (1930) with *Phaseolus aureus* are decidedly not comparable with the present work, due to a number of technical difficulties such as crowding of the seedlings, insufficient thermal adaptation, and control; these have been discussed by Crozier and Navez (1930-31). In Harvey's recent book (1930, p. 327) there are given some data for wheat seedlings (without any experimental detail). A plotting of his data between 0 and 25° shows a critical temperature at 11° with a temperature characteristic of 12,550 ± calories above and 17,150 ± calories below that temperature. Above 25° the relation no longer holds. Since the technic was not given, and the temperature intervals are too great, little significance can be attached to these values. However, they do show a striking agreement with many of the existing values for respiratory process. Navez (1928-29) with *Vicia faba* reported a temperature characteristic of 16,250 between 7.5° and 20°. Crozier and Navez (1930-31) obtained a value of 16,500 for *Phaseolus aureus* between 12° and 20°. These values are in accord with the one reported here for *Lupinus albus* above the critical temperature only. Whether the

difference between the values obtained here and the values obtained by Navez and Crozier and Navez is due to the difference of the seeds used or due to the difference in the stages of development we are not yet in a position to ascertain, but these values have all been observed before in experiments of this nature (*cf.* Crozier, 1924-25).

Table I summarizes the temperature characteristics for both the oxygen consumption and production of CO₂ by *Lupinus albus* and *Zea mays*.

In going over the figures in Table I it is at once apparent that the temperature characteristics obtained with the same seeds at the same developing stage are not necessarily the same with respect

TABLE I

Summary of Temperature Characteristics for the Oxygen Consumption and Production of CO₂ of Lupinus albus and Zea mays

	Critical temp. (C.T.), °C.		Value of μ below C.T.		Value of μ above C.T.	
	O-cons.	CO ₂ - prod.	O-cons.	CO ₂ -prod.	O-cons.	CO ₂ -prod.
<i>Lupinus albus</i>	19.5	20.0	16,600	24,000	(11,700)*	16,100
<i>Zea mays</i>	19.5		21,050	20,750	(13,100)*	20,750

* Since the lines were drawn through a limited number of points, these values cannot be given much weight.

to oxygen consumption and production of CO₂. For *Lupinus*, the critical temperatures for oxygen consumption and for production of CO₂ come at very nearly the same place (20°), but the respective temperature characteristics are quite different. For oxygen consumption it is 11,700 calories above the critical temperature, and 16,100 calories below; the latter agrees with that for production of CO₂ above the critical temperature, but not below. For *Zea*, the temperature characteristic for production of CO₂ is of the same order of magnitude as that for oxygen consumption below the critical temperature (20°). Such a temperature was not found in the case of production of CO₂. Above the critical temperature the value of μ is 13,100 for oxygen consumption.

These differences are rather striking, considering the fact that the

seeds are germinated alike and are from the same lots. Two interpretations suggest themselves. The first is that if temperature characteristics indicate in any way the mechanism of the chemical reactions involved in respiration, then such differences in the values obtained would suggest that the mechanisms of the reactions involved in the two phases of respiration—oxygen consumption and production of CO₂—may not necessarily be the same, and may be quite independent of one another, a concept not entirely novel to students of plant respiration.

A corollary of the above is that since the temperature characteristics for rates of oxygen consumption and production of CO₂ are different, the ratio of the two—the respiratory quotient—should be a function of temperature. Superimpose two lines of different slopes so that they intersect at a point corresponding to a given temperature with reference axes similar to those in Fig. 1, and the R.Q. at that temperature is 1; at temperatures above and below that point the values of R.Q. should be either greater or less than 1, according to the slopes of the lines. Such a picture is merely qualitative,—nevertheless the fact is significant.

A perhaps serious objection which may be made against this interpretation is supplied by the second alternative suggestion as to the reason for the differences between μ 's for consumption of O₂ and production of CO₂ by the same seeds. This is the fact that although the seeds are from the same lots and are treated in the same way with respect to conditions of germination, the technic with which the rate of oxygen consumption was obtained is different from that used in ascertaining the rate of production of CO₂. In the former case the seeds were in a closed moist chamber, while in the latter case the seeds were subjected to a rather rapid stream of moist air. If this difference does introduce an effect determining the apparent temperature coefficient, however, it is difficult to account for the identical values of temperature characteristics for oxygen consumption and production of CO₂ below the critical temperature in the case of *Zea*; likewise it is difficult to account for the occurrence of the critical temperatures for the two processes at so very nearly the same place in *Lupinus*. Before any definite conclusion can be drawn this point must be more carefully tested, preferably with single seeds in both cases.

SUMMARY

The rates of production of CO_2 by germinating seeds of *Lupinus albus* and *Zea mays* were studied between temperatures 12.5° and 25°C . with the HCl-Ba(OH)_2 titration method. The temperature characteristics found are different from those previously obtained for the oxygen consumption of the same seeds germinated in the same manner. For *Lupinus*, the temperature characteristics above and below the critical temperature of 20° are $16,100 \pm$ and $24,000 \pm$ calories respectively. For *Zea*, no evidence of a critical temperature was found in this region, and the temperature characteristic is $20,750 \pm$ calories throughout the range of temperature tested. The possible interpretations of the difference in the values of temperature characteristics for oxygen consumption and for production of CO_2 are noted.

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THE RÔLE OF PHOSPHATE IN BIOLOGICAL OXIDATIONS

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Although it has been demonstrated repeatedly that phosphates exercise a catalytic effect in biological oxidations, no one has successfully identified the particular ionic species to which phosphate catalysis is due. In consequence of observations on the stimulus to carbon dioxide production exerted by phosphate solutions on *Elodea canadensis*, Lyon (1) concluded that the PO_4^{\equiv} ion was the active form. He succeeded in demonstrating that the results could conveniently be expressed by the hyperbolic equation $(\text{CO}_2 - a)(\text{pPO}_4^{\equiv} - b) = K$. Furthermore, reference to the literature seemed to indicate that for lipase in phosphate buffers the following similar relationship was obtained:

$$(\text{Activity of enzyme})(\text{pPO}_4^{\equiv})^n = K$$

where $\text{pPO}_4^{\equiv} = -\log(\text{PO}_4^{\equiv})$. This direct proportionality between enzyme activity and PO_4^{\equiv} ion concentration would seemingly be an acceptable proof of the catalytic activity of this ionic species.

It happens, however, that other considerations, which the author fully appreciated, render this proof somewhat less convincing. In the first place it is necessary to bear in mind that in almost all of the experiments cited, the PO_4^{\equiv} ion concentration was changed, not by additions of phosphate at constant pH, but by alteration of the hydrogen ion concentration. In consequence effects attributed to the PO_4^{\equiv} ion are inextricably associated with the powerful action of the hydroxyl ion.

The single exception to this method of altering the PO_4^{\equiv} ion concentration was presented as a citation from experiments by Platt and Dawson on pancreatic lipase (2). Here the authors increased the PO_4^{\equiv} ion concentration by additions of phosphate at constant pH, a method which eliminates any effects due to changing hydrogen ion activity. Unfortunately, however, one is not enabled by this means

to increase only the $\text{PO}_4^{=}$ ion concentration. Over the range of acidity usually investigated a change in the concentration of any one of the phosphate ions would necessarily be associated with proportional increases in the remainder. This is apparent from the following equations

$$[\text{H}^+] = \frac{K_1 [\text{H}_3\text{PO}_4]}{[\text{H}_2\text{PO}_4^-]} = \frac{K_2 [\text{H}_2\text{PO}_4^-]}{[\text{HPO}_4^-]} = \frac{K_3 [\text{HPO}_4^-]}{[\text{PO}_4^{=}]}$$

In consequence hyperbolic relationships corresponding to

$$(\text{Activity of enzyme}) (\text{pPO}_4^{=})^n = K \quad (1)$$

can be obtained with any one of the other ionic species under consideration. For example, it may be shown by derivation from Equation 1 that

$$(\text{Activity of enzyme}) (\text{pK}' + \text{pHPO}_4^-)^n = K \quad (2)$$

where $K' = \frac{K_3}{[\text{H}^+]} = \text{a constant}$

$$K_3 = \frac{[\text{H}^+] [\text{PO}_4^{=}]}{[\text{HPO}_4^-]}$$

and $\text{pHPO}_4^- = -\log [\text{HPO}_4^-]$

It is therefore apparent that if the data conform to Equation 1, they agree also with Equation 2. We are, in consequence, unable to resort to these equations to identify the particular ionic species to which the catalytic effect of phosphate is due.

Furthermore, we are reminded by Dawson and Platt (3) that the hyperbolic relationship just described is true of pancreatic lipase only under certain limited conditions. Serious deviations are observed in fluids more alkaline than pH 7.2.

To determine the mechanism of phosphate catalysis fairly simple systems are clearly needed. The data of respiration experiments are singularly difficult to analyze because of the unknown nature and concentration of the substrate, the participation of other catalysts (enzymes), and uncertainty about the diffusion of phosphate ions into the cell. We have endeavored to investigate the problem by having

recourse to quite a simple reaction,—the oxidation of glyceric aldehyde by phenolindophenol or other suitable dyestuff. The experiments were conducted at constant hydrogen ion activity in the absence of enzymes and under conditions which permitted precise electro-metric observations of the rate of oxidation.

As is indicated in the following paragraphs, we agree with Lyon in attributing phosphate catalysis to the $\text{PO}_4^{=}$ ion, even though we have been but partly convinced by the evidence presented by him.

EXPERIMENTAL

In the first group of experiments the oxidation of glyceric aldehyde (0.0044 M) at different hydrogen ion activities was studied. Methylene blue, 1-naphthol-2 sulfonate indophenol, and phenolindophenol, all 0.00018 M, were used as the oxidizing agents. The hydrogen ion activity was maintained by the use of phthalate, borate, carbonate, and phenylalanine buffers, in each case the buffer concentration being 0.1 M. In those tests in which phosphate was added, it was used in a concentration of 0.05 M.

Controls were also run which were identical with the experimentals except that the substrate, glyceric aldehyde, was omitted.

The experiments were conducted in small soft glass bulbs of about 5 cc. capacity. Each bulb after being blown was drawn off from the parent tube in such a way as to produce a long, slender, capillary outlet. While still hot, the open end was placed in the appropriate buffer and allowed to fill partly on cooling. The bulbs were then boiled to displace all the air with steam. While the contents were still boiling the bulbs were inverted and the buffer expelled. The open ends were promptly plunged into the buffered, glyceric aldehyde—dye solutions. The tiny air bubble which remained in each bulb on cooling was expelled by warming. The tubes were immediately sealed, the bulbs suspended in a bath at $31 \pm 0.02^\circ$, and the time noted. Completion of oxidation was determined by noting the time required for decoloration of the contents. For experiments which have to run many hours, we have found these bulbs superior to vacuum tubes.

The results are presented in Fig. 1. Wherever the pH is expressed to three decimal places the quinhydrone electrode and type K potentiometer were used. In a few instances where the amount of solution

available was small, the pH was determined colorimetrically and expressed to the first decimal place.

The results obtained with methylene blue and phenolindophenol demonstrate that phosphate exercises no catalytic effect at pH 4.77. This conclusion has to be qualified by mention of the aberrant behavior

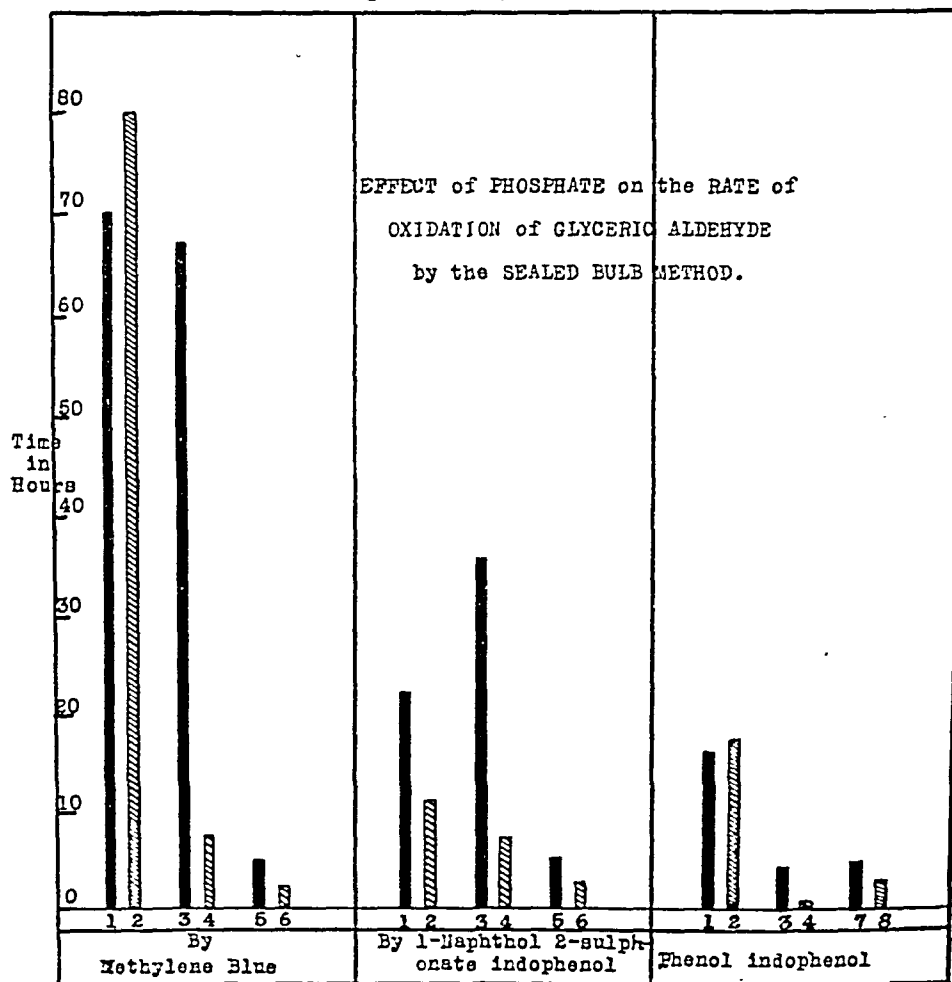


Fig. 1. 1. 0.1 M phthalate pH 4.774
 2. 0.1 M phthalate, 0.05 M phosphate pH 4.769
 3. 0.1 M borate pH 7.861
 4. 0.1 M borate, 0.05 M phosphate pH 7.852
 5. 0.1 M phenylalanine pH 7.8
 6. 0.1 M phenylalanine, 0.05 M phosphate pH 7.8
 7. 0.1 M carbonate pH 7.8
 8. 0.1 M carbonate, 0.05 M phosphate pH 7.8

of naphthol sulfonate indophenol in which catalysis was observed. It is supported, however, by the early work of Löb and colleagues (4, 5) and Witzemann (6) in which the oxidation of glucose by hydrogen peroxide was catalyzed but slightly by acid phosphate solutions. Undissociated phosphoric acid and the H_2PO_4^- ion are therefore eliminated from consideration.

At pH 7.8 to 7.9, in all buffers, phosphate clearly catalyzed the oxidation. The most pronounced effects were observed in borate, in which phosphate additions reduced, in striking fashion, the time of oxidation. As an incidental finding attention should be drawn to the very obvious inverse relationship between the time of oxidation in the borate controls and the oxidation-reduction potential of the dye. This relationship, though not unexpected, was not observed in the phenylalanine controls. We recognize the possibility that phenylalanine may itself have suffered oxidation, and in the continuation of this research we propose to inquire more fully into this question.

In the next group of experiments an exact quantitative study of the phosphate effect was made. Phenolindophenol, in an initial concentration of 0.00037 N, was used as the oxidizing agent in borate buffers (0.1 M) at pH 7.83 to 7.92. The total amount of phenolindophenol initially present was 10×10^{-6} mols or 20×10^{-6} equivalents. The oxidation was followed electrometrically by potential measurements on the system $\text{Pt} / \frac{\text{Oxidized dye}}{\text{Reduced dye}} / \text{KCl sat.} / \frac{\text{Sat. KCl}}{\text{HgCl}} \text{ Hg}$. In every experiment duplicate readings were made by the use of two electrodes of bright platinum. A titration cell almost identical with that described by Clark (7) was used. In it was placed a solution of the dye in the desired buffer. The solution of glyceric aldehyde was placed in an aeration vessel and both solutions were thoroughly de-aerated by washing with oxygen-free nitrogen.¹

¹ Nitrogen was freed from oxygen by passing it over tightly rolled copper gauze in a combustion furnace at a temperature of 650 to 700°. Never more than half the length of copper was allowed to become oxidized. It was then reduced with hydrogen. The gas before entering the cell was passed through a wash bottle of distilled water and a spray trap, both in the thermostat. All joints were gas tight. Rubber tubing, which commonly permits oxygen leakage, was not used. Mercury seals, deKhotinsky cement seals, and fused glass joints were used throughout.

Several readings were then made on the buffered dye solution in the cell. 5 cc. of deaerated glyceric aldehyde solution (containing 2.45×10^{-5} mols) were added and further readings made at intervals

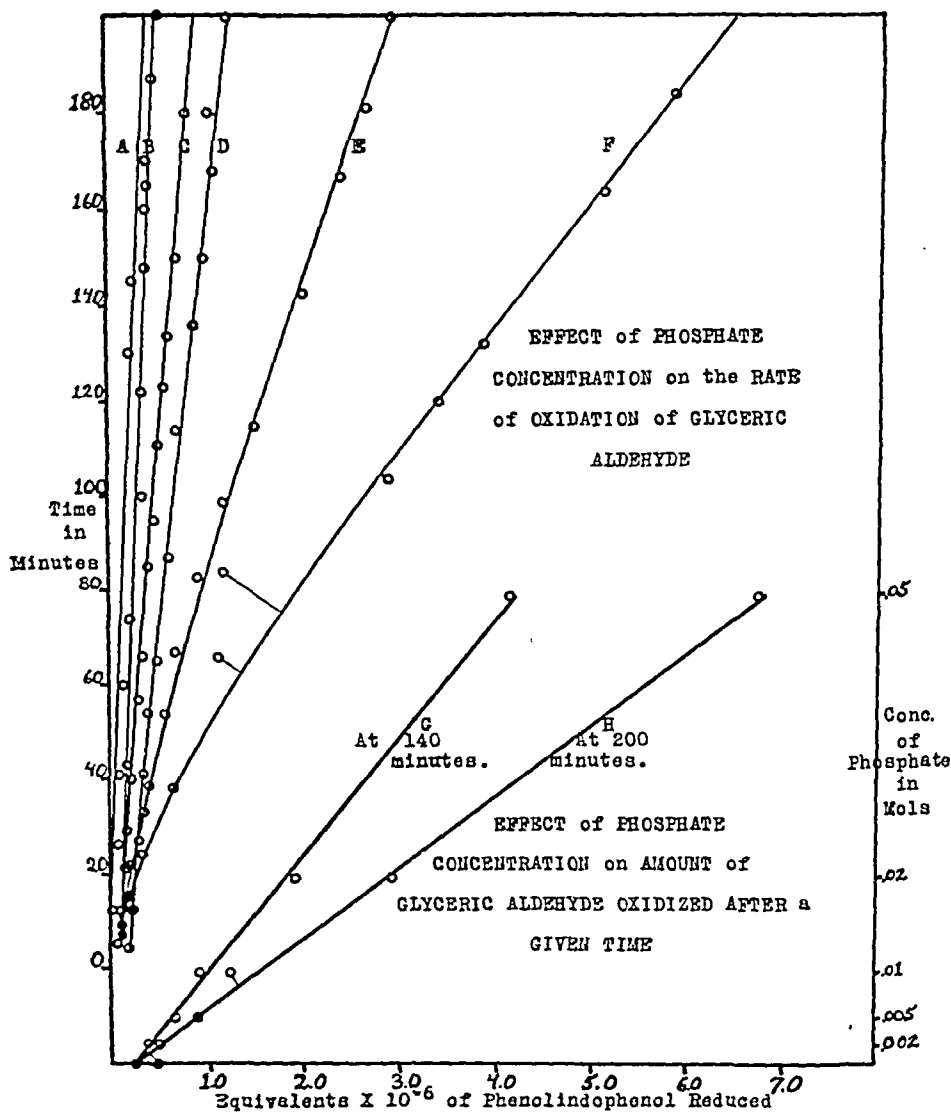


Fig. 2. A. Borate, 0.1 M; phosphate, 0.000 M; pH 7.833
 B. Borate, 0.1 M; phosphate, 0.002 M; pH 7.921
 C. Borate, 0.1 M; phosphate, 0.005 M; pH 7.913
 D. Borate, 0.1 M; phosphate, 0.010 M; pH 7.900
 E. Borate, 0.1 M; phosphate, 0.020 M; pH 7.889
 F. Borate, 0.1 M; phosphate, 0.050 M; pH 7.833

of 15 minutes or so for about 4 hours. From these readings the equivalents of dye reduced were calculated.²

The results are plotted in Fig. 2, Curves A to F. When the equivalents of dye reduced in a given time are plotted against phosphate concentration (Fig. 2, Curves G and H), it is to be observed that the relationship is a linear one.

This observation now permits us to differentiate between the HPO_4^- ion and the $\text{PO}_4^{=}$ ion in an attempt to identify the ionic species possessed of catalytic activity. At pH 7.8 to 7.9 most of the phosphate is present as HPO_4^- but a small amount exists as $\text{PO}_4^{=}$. The concentration of the latter may be calculated from the equation:

$$\frac{[\text{H}^+]^3 [\text{PO}_4^{=}]}{K_1 K_2 K_3} + \frac{[\text{H}^+]^2 [\text{PO}_4^{=}]}{K_2 K_3} + \frac{[\text{H}^+] [\text{PO}_4^{=}]}{K_3} + [\text{PO}_4^{=}] = C$$

where K_1 , K_2 , K_3 are the dissociation constants of phosphoric acid and C the total molar concentration of H_3PO_4 and the three ionic species. The concentration of HPO_4^- is practically equal to total phosphate. The initial concentration of glyceric aldehyde was 0.000089 M. Considering now phosphate solutions of 0.02 M it follows that the ratios

$$\frac{[\text{HPO}_4^-]}{[\text{glyceric aldehyde}]} \quad \text{and} \quad \frac{[\text{PO}_4^{=}]}{[\text{glyceric aldehyde}]}$$

possess the values 225 and 0.055 respectively.³ The former is already so large that it is difficult to understand by reference to any acceptable

² The equation given by Clark (8) is

$$\begin{aligned} E_{\text{A30}^\circ} &= E_0 - 0.03006 \log \frac{S_r}{S_0} + 0.03006 \log [K_1 K_2 + K_1 \text{H}^+ + (\text{H}^+)^2] \\ &\quad + 0.03006 \log [\text{H}^+] - 0.03006 \log [K_0 + (\text{H}^+)] \\ E_0 &= +0.6494 \quad K_1 = 3.6 \times 10^{-10} \\ K_0 &= 8.0 \times 10^{-9} \quad K_2 = 2.3 \times 10^{-11} \end{aligned}$$

These constants were assumed to be the same for 31° as for 30°. The numerical factor 0.03006 was changed to 0.03015. The potential of the calomel cell at 31° was +0.2432. After calculating the potential E_r' , at the given pH, of the system containing equal amounts of reduced and oxidized dye the determination of the amount of reduction was taken from a curve in which per cent reduction was plotted against $(E_0 - E_r')$, where E_0 M. F. observed $\approx E_0 + E_{\text{calomel cell}}$.

³ In calculation of $\text{PO}_4^{=}$, the tables by Holt, La Mer, and Chown (9) were employed.

theory of catalysis why further additions of $\text{HPO}_4^=$ should continue to give a proportional increase in oxidation. On the contrary, the $\text{PO}_4^=$ ion concentration relative to that of the substrate is so small that it is readily understandable why a two or three fold increase in $\text{PO}_4^=$ concentration should provoke a similar increase in substrate oxidation if to the $\text{PO}_4^=$ ion be attributed the catalytic activity of the system. We conclude therefore that these results are best interpreted by regarding the $\text{PO}_4^=$ ion as the catalyst. This conclusion is based on the premise that the disodium and trisodium phosphates are so completely dissociated that the possibility of molecular salt catalysis can be ignored. Professor V. K. La Mer suggests that the linear relationship between phosphate concentration (at constant pH) and the rate of oxidation is very likely an example of general basic catalysis by $\text{PO}_4^=$ ion in the sense used by Brönsted (10-13). We propose to investigate the subject from this point of view.

SUMMARY

1. The effect of phosphate on the oxidation of glyceric aldehyde by methylene blue, 1-naphthol 2-sulfonate indophenol, and phenol-indophenol has been studied.

2. At pH 4.77 in a phthalate-buffered medium phosphate does not catalyze the reaction.

3. At pH 7.9 in solutions buffered with borate, carbonate, or phenylalanine marked catalysis by phosphate is observed. The effect is most pronounced in borate.

4. Phosphate catalysis, within the limits studied, is strictly a linear function of the phosphate concentration.

5. The high concentration of $\text{HPO}_4^=$ and the low concentration of $\text{PO}_4^=$ relative to that of the substrate virtually demand the conclusion that the $\text{PO}_4^=$ ion is the active catalytic species.

We wish to express our thanks to Professors H. A. Spoehr and R. T. Woodyatt for samples of pure glyceric aldehyde and to Professor V. K. La Mer and Dr. H. H. Strain for a number of valued suggestions.

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THE INSENSITIVITY OF PARAMECIUM TO CYANIDE AND EFFECTS OF IRON ON RESPIRATION

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Some recent work, notably that of Dixon and Elliott,¹ has indicated that not all cells and tissues are completely cyanide-sensitive in the sense that they reduce their rate of respiration in the presence of cyanides. Allen² has pointed out that not all the respiratory exchange in *Planaria* may be inhibited by cyanides. This has indicated that there may be other oxidation systems in the cells besides those affected by cyanides. Lund³ found some years ago that KCN had little or no effect on oxygen consumption by *Paramecium*. We proposed to reinvestigate this question and to determine if iron is present in the protoplasm of *Paramecium*, acting as the respiration catalyst according to the theory of Warburg, and to note if addition of iron would cause an increase in the rate of respiration in the *Paramecium*.

All of the present experiments have involved the measurement of oxygen consumption in the Thunberg-Winterstein microrespirometer. Using the type of respiratory vessel illustrated in Fig. 1, it was a simple matter to remove or add organisms in various culture media and solutions. In all experiments a preliminary determination was made of the rate of oxygen consumption before cyanide was added to the sample of *Paramecium*. The comparative rates of oxygen consumption indicated the intensity of total oxidation within the cells under experimental conditions.

To test the apparatus and the methods employed, two experimental runs were made on the same sample of *Paramecium* suspended in tap water. It was found that they consumed the same amount of oxygen over the same period of time. When 1 cc. of a 2 cc. sample of organisms was removed, and replaced with 1 cc. of distilled water without organisms, the respiration rate of the sample was re-

¹ Dixon, M., and Elliott, K. A. C., *Biochem. J.*, 1929, 23, 812-830.

² Allen, G. D., *Am. J. Physiol.*, 1919, 48, 93.

³ Lund, E. J., *Am. J. Physiol.*, 1918, 45, 365.

duced 50 *per cent*, approximately one-half of the total number of cells being removed by this method. When a sample of *Paramecium* was removed after a normal run, washed by centrifuging in distilled water, and replaced in the respirometer, no change in the respiratory rate was observed. It was perfectly safe to remove experimental samples from the apparatus, wash them in a solution, and replace in the apparatus, without significant loss of cells. The actual experiments carried through in this way were divided into two groups: (1) those in which the organisms were not suspended in distilled water nor tap water, but were in culture media with a normal food supply, and (2) those in which the cells were washed in distilled water to remove food supply and allow the organisms to come to a "basal" state for experimental purposes.

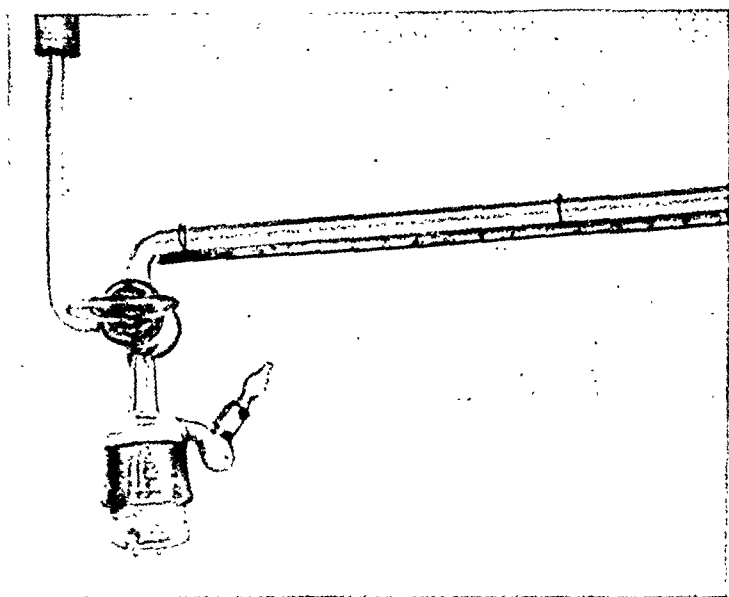


FIG. 1. Type of respiration chamber used in the microrespirometer

In the first group of experiments determinations of the oxygen consumption of samples of *Paramecium* in culture media were made, then the cells were centrifuged out of the media, suspended in KCN solutions of different strengths, and experimental determinations made of the effects of the KCN on the oxygen consumption. In all such cases there were reductions in the rate of respiration from 10 per cent to 50 per cent, when the medium was replaced by cyanide concentrations of $M/200$, $M/300$, $M/500$, $M/1000$, $M/2000$, $M/4000$, $M/5000$, or $M/10,000$.

It is interesting to note in this connection that Lund³ points out that the rate of oxidation in the cell decreases simultaneously with the disappearance of available food. When the *Paramecium* in the above experiments were placed in the solutions of pure cyanide they were deprived of their food supply. It was possible

that this might be the real cause of the pronounced decrease in the respiration rate, rather than the actual effect of the KCN. The reduction in the oxygen consumption was indicated not to be produced by the action of KCN by the following experiments:

The respiration rate of samples of *Paramecium* in culture media was taken and then the same samples were washed with distilled water, suspended in the same volume of distilled water as the culture media they were previously in, and respiratory determinations again made on the organisms freed of their natural food. In such cases there was an average reduction of respiration of near 44 per cent in the absence of nutrient media. On further washing and replacement of the distilled water with an equal volume of $M/4000$ KCN, no further decrease in the respiratory rate was observed, but actually an increase of 6 per cent over the run in distilled water where food was absent. All of the cells remain in a normal condition and retain their activity with such treatment. It can be seen that the presence or absence of a nutrient medium had the marked effect on oxygen consumption, whereas the KCN had little or no effect.

All of the later and significant experiments were performed on *Paramecium* in which the normal preliminary determination of oxygen consumption was made in the absence of culture media, with washed *Paramecium* suspended in distilled water or in phosphate buffer solutions, followed by experimental runs on the identical sample of organisms in the KCN solutions of the concentrations used. The total results from this group of experiments showed a slight increase in the amount of oxygen consumed in the presence of buffered or unbuffered KCN solutions, rather than the marked decrease in oxygen consumption expected on the basis of experiments with other organisms and tissues.

Paramecium allowed to stand in $M/1000$ KCN solutions for 4 hours before being tested for respiratory rate, still showed no decrease in their rate of oxygen consumption as measured in the microrespirometer. In all the experiments performed, no decrease in the respiration rate was observed in any concentration of KCN from $M/200$ to $M/10,000$. In fact most experiments indicated a slight increase in the amount of oxygen consumed in the presence of the cyanide.

The average pH of the culture media in which the *Paramecium* were grown was found to be 7.3. The pH of a $M/5000$ KCN solution is about 8.8, and of a $M/1000$ KCN solution 9.7, an increase in alkalinity that is quite marked in unbuffered solutions.⁴ The question arose

⁴ Cf. Bodine, J. H., *J. Gen. Physiol.*, 1924, 7, 19-23.

regarding the effect of pH on the oxygen consumption of the *Paramecium*. Buffer mixtures of KH_2PO_4 and Na_2HPO_4 adjusted to a pH of 7.3 in M/100 concentrations held added KCN of high concentration in a constant pH range, varying only 0.1–0.2 pH in electrometric measurement. In all cases when these buffer mixtures were substituted for distilled water or tap water as the suspension medium, no effect was observed on the addition of KCN of various concentrations in any way differing from that when the protozoa were sus-

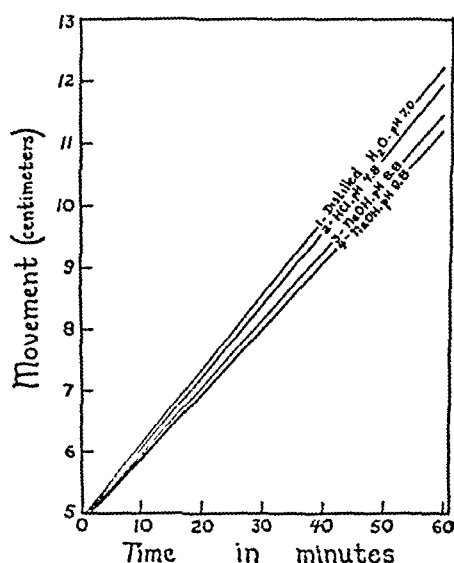


FIG. 2. Effect of HCl and NaOH on the respiration rates of a sample of *Paramecium*.

pending in an unbuffered non-nutrient medium. These experiments agree with those of Lund,⁵ who added hydrochloric acid to his cyanide solutions.

The alkalinity of a pure KCN solution does not seem to affect *Paramecium*. Experiments performed with these organisms in the presence of NaOH at pH values from 8.0 to 9.8 caused an average 11 per cent decrease in the rate of oxygen consumption under that observed in distilled water. These were the pH values of some unbuffered KCN solutions used. This seems to add strength to the assumption that a change to an alkaline condition comparable to that

⁵ Lund, E. J., *Am. J. Physiol.*, 1921, 57, 336.

found in pure cyanide solutions does not inhibit or affect to a marked degree the action of the cyanide. The NaOH solutions really cause a slight decrease in the oxygen consumption while pure cyanide caused a slight increase in oxidation rate at the same pH values. We are of

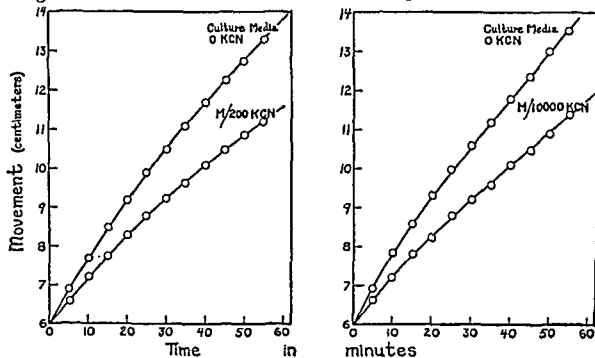


FIG. 3. Slight effect of different concentrations of KCN on oxygen consumption (explanation in text).

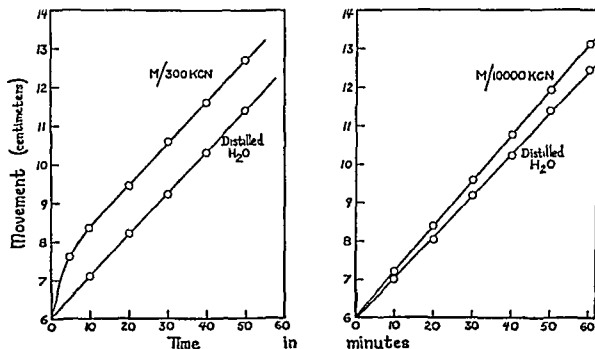


FIG. 4. Lack of inhibition of respiration by potassium cyanide (explanation in text).

the opinion that it is unnecessary to strictly control the hydrogen ion concentration of the media surrounding the *Paramecium* in order to obtain an indication of the insensitivity of this protozoan to cyanide. In Fig. 2 is shown the result of a series of experiments on the effects of hydrochloric acid and sodium hydroxide on the oxygen consumption of a sample of *Paramecium*. The region of change of the rate of oxygen consumption with change in pH is very narrow indeed. We have found that acid solutions are much more injurious to *Paramecium* than alkaline substances, a condition not encountered when using KCN.

Fig. 3 shows the results of two interesting experiments on two samples of *Paramecium* with nearly the same number of organisms in each sample. The preliminary determination of oxygen consumption in each case was made with the cells in culture media. This was removed and replaced with KCN solutions of $\text{M}/200$ concentration in one experiment and $\text{M}/10,000$ concentration in the second. Here it is interesting to note that even in widely differing concentrations of cyanide, the percentage reduction of oxygen consumption remains nearly identical, *i.e.*, 26 per cent reduction in the one case and 29 per cent reduction in the other.

It can be shown, as mentioned above, that if the natural medium is first removed from the organisms, and they are suspended in a buffered medium or in distilled water, there will be no resulting reduction in respiration on the addition of KCN whatever, but instead, a slightly increasing rate of respiration over a short period. The whole difference in the rates of oxygen consumption in the two experiments is apparently due to the amount of available food material or to the effect of a changed immersion medium, and in no way connected with inhibition by cyanide. An example of such a condition is indicated in Fig. 4. In each case the normal rate of respiratory activity is approximately the same for the two samples of organisms suspended in distilled water. On the addition of KCN in the indicated concentrations ($\text{M}/300$ and $\text{M}/10,000$) there was no decrease in the oxygen consumption. One notices instead a temporary increase in the oxygen consumption on the addition of $\text{M}/300$ KCN. We are inclined to disregard the first portion of this curve, on the assumption that equilibrium had not been established in the apparatus, and to regard the straight por-

tion of the curve as significant. It will be noted that no decrease in respiratory activity is indicated during nearly an hour of exposure of the cells to the high concentration of KCN. Similarly, on replacing the distilled water with $M/10,000$ KCN, no decrease in oxygen consumption was indicated, but instead a slight increase comparable to the effects of low concentrations of pure non-toxic salts.

In Fig. 5, the relative effects of the presence or absence of a nutrient medium may be compared with the effect on total oxygen consumption of $M/4000$ KCN when used as the immersion medium. It

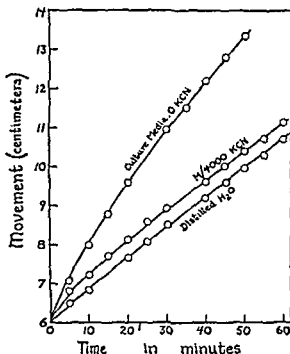


FIG. 5. Comparative effects of nutrient and non-nutrient media on the oxygen consumption of a sample of *Paramecium*, together with absence of inhibition of respiration by KCN (explanation in text).

will be seen that removal from a nutrient medium to a non-nutrient one produces a decrease in the rate of oxygen consumption in these protozoa, and addition of $M/4000$ KCN instead of the non-nutrient medium has no inhibitory effect.

We have found that sometimes, in a completely unbuffered medium, cyanide of the concentration of $M/200$ will injure and rupture the protozoan cell, and always that great injury results if concentrations of $M/100$ and higher are used. This rupture with death of the cells results in a decrease in the oxygen consumption of the sample of organisms, but it is reasonable to expect that if the cyanide is acting

in any way as a true respiratory poison, it would inhibit the respiration of the cells without their rupture, and at concentrations showing no inhibitory effect whatever in these experiments. Gerard and Hyman⁶ have recently found *Paramecium* relatively insensitive to NaCN in high concentrations, to much the same extent as we find for KCN.

We have found it practically impossible to demonstrate the actual penetration of KCN into the cells of *Paramecium* when in low concentrations of cyanide. There is absolutely no change in the microscopic appearance of the cells, but only an increased activity and an occasional increase in vacuole formation. It is an interesting fact that although KCN in concentrations greater than M/200 causes swelling of the cells and seems to enter them, there is no reduction in the respiratory activity of the organisms until the cells are actually ruptured and destroyed, whereupon respiration completely ceases. Lund (1921) has pointed out that cytolized and disintegrated *Paramecium* always cease to show respiratory activity perfectly independently of the presence or absence of cyanide. Further work is now under way to determine the extent and speed of the penetration of KCN into the *Paramecium* cell.

Effects of Iron Compounds

The experimental indications have been set forth in this paper that *Paramecia* are stable with respect to their oxygen consumption in the presence of KCN. Some evidence exists that cells sensitive to cyanide (*Paramecium* seem to be insensitive) show an increase in their oxygen consumption in the presence of iron, particularly iron in the form of more soluble iron salts which may be capable of augmenting the respiration-catalyst in the cell, if iron may be considered as such. We have conducted a series of experiments to determine the effects of various concentrations of ferric oxide, ferrous chloride, ferric sulfate, ferrous phosphate, ferrous nitrate, and ferrous ammonium sulfate, on the rate of oxygen consumption in *Paramecium*. These substances were prepared in solutions or suspensions in distilled water. Careful check was made of the pH values of the solutions added to the organisms in the microrespirometer, replacing the distilled water or

⁶ Gerard, R. W., Private communication, 1931.

tap water suspension medium in which preliminary determinations of oxygen consumption were made.

Table I summarizes and presents the data from these experiments on iron compounds. Ferric oxide, being insoluble in water, was taken into the gullet of every *Paramecium*, and became concentrated in vacuoles, sometimes accumulating to so great an extent as to alter the movement of the cell in swimming about. In high concentrations ($m/1000$) the cells were killed and ruptured, with consequent loss in respiratory rate. In lower concentrations ($m/5000$) of ferric oxide, a reduction of respiratory rate could be obtained from 10 to 30 per cent. On being returned to distilled water, the organisms recovered their normal rate of oxygen consumption. Several interesting experiments in this connection involved replacement of the $m/5000$ ferric oxide with $m/1000$ KCN, whereupon the protozoa returned to their normal respiratory rate, perfectly independent of the presence of the KCN, as though the medium contained no foreign substance whatever. There was no tendency for the ferric oxide taken in by the cell to counteract the lack of effect of cyanide, and both of these substances act in the same manner as when used separately. It is the opinion of some workers that iron and cyanide act as antagonists to each other but no indication of this effect is apparent in these experiments.

It is an interesting fact that $m/5000$ ferric oxide produces the same reduction in the rate of respiration whether in a buffered or an unbuffered solution, so long as approximately the same number of cells are present.

Ferrous chloride ($FeCl_2$) is extremely toxic for *Paramecium*. The organisms live for only a few seconds in an $m/1000$ solution. $m/10,000$ solutions of ferrous chloride produce marked reductions in the rate of respiration with no apparent effect on the microscopic appearance of the cells. Ferrous chloride solutions are quite acid in reaction ($m/5000$ $FeCl_2 = pH\ 4.7$), but experiments were conducted with *Paramecium* in distilled water and then in HCl of pH 4.8 without change in oxygen consumption from the normal rate in the solution near neutrality. If however, solutions of ferrous chloride are neutralized with phosphate buffer, they have less effect in lowering the oxygen consumption of *Paramecium*. In a similar manner, on addition of NaOH to solutions of ferrous chloride, neutral solutions could be produced

which did not markedly alter oxygen consumption, although large quantities of ferrous chloride were present. In these cases heavy precipitates were produced, probably insoluble ferric hydroxide that

TABLE I

Substance	Solubility	Concentration	pH	Change in O ₂ -cons.	Effects on cells	Condition of cells
				per cent		
Ferric oxide Fe ₂ O ₃	Insol. in H ₂ O	M/ 1,000	5.9	-62	Sl. toxic	Swollen or killed
		M/ 5,000	6.3	-32	No effect	Normal
		M/10,000	6.5	-30	No effect	Normal
Ferrous chloride FeCl ₂	160.1 ¹⁰⁰ in H ₂ O	M/ 1,000	4.3	—	Toxic	All killed
		M/ 5,000	4.7	-60	Toxic	Swollen and ruptured
		M/10,000	4.9	-43	No effect	Normal
Ferric sulfate Fe ₂ (SO ₄) ₃	Very sol. in cold H ₂ O	M/ 1,000	2.4	-100	Toxic	Killed instantly
		M/ 5,000	2.8	-100	Toxic	Killed
		M/10,000	3.2	-100	Toxic	Killed in 2 min.
Ferrous phosphate Fe ₃ (PO ₄) ₂	Insol.	M/ 5,000	5.9	-43	No effect	Normal
Ferrous nitrate Fe(NO ₃) ₂	200.° in cold H ₂ O	M/ 5,000	3.4	-75	Toxic	Many dead. No cells ruptured
Ferrous ammonium sulfate FeSO ₄ (NH ₄) ₂ SO ₄	18.° in cold H ₂ O	M/ 1,000	4.6	—	Toxic	All killed
		M/ 5,000	5.0	None	No effect	Normal
		M/10,000	5.6	None	No effect	Normal
		M/20,000	5.8	None	No effect	Normal

could not be taken into the cells and produce reduction in respiratory activity.

Ferric sulfate was found to be extremely toxic for *Paramecium*, killing the organisms within a few minutes in concentrations as low as M/10,000. This salt is very soluble and produces an acid solution. *Paramecium* exposed to the action of very dilute ferric sulfate, move

about rapidly until death. When their activity ceases they show no external change of form, but have a larger distribution of opaque particles within the cell.

Ferrous phosphate was found similar to ferric oxide and ferrous chloride in its reduction of respiration in *Paramecium*. The cells became inactive in the presence of this salt with no change in microscopic appearance.

Ferrous nitrate is quite toxic for *Paramecium*. $m/5000$ concentration causes marked reduction in respiratory activity (75 per cent reduction) with death of the cells within 1 hour.

Paramecium live for about 30 minutes in $m/1000$ ferrous ammonium sulfate and become enlarged and ruptured. In $m/5000$ solution of this complex salt, a 1 per cent increase in oxygen consumption over the normal in distilled water may be obtained. $m/10,000$ and $m/20,000$ ferrous ammonium sulfate produce no significant change in oxygen consumption, the whole action of this substance being scarcely perceptible. These results agree with those of Rosenthal and Voegtlin⁷ with respect to this particular salt on the respiration of rat tissue. In no instance did they obtain an increase in oxygen consumption by addition of iron compounds to the tissue. For the *Paramecium* cell simple iron salts appear to be toxic in proportion to their solubility and in about the same manner as other heavy metal salts on most cells.

Following the method for the detection of minute amounts of iron in biological material given by Elvehjem and Hart,⁸ we have attempted to obtain an indication of iron in the protoplasm of *Paramecium*.⁹ Comparison of the color reaction in the *Paramecium*-ash solution with that in a standard control solution containing 0.1 mg. of iron, showed the faintest tint in contrast with the deep red of the standard control.

⁷ Rosenthal, S. M., and Voegtlin, C., *Pub. Health Rep.*, 1931, 46, 521.

⁸ Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1926, 67, 43.

⁹ The method involved the evaporation of a mass of *Paramecium* (collected from their culture media by centrifuging) of 0.5 gm. to dryness, ashing, the taking up of the ash in dilute HCl, filtering, and treating the filtrate with 40 per cent NaOH, boiling for 1 hour. When hydrolysis was complete, the solution was made acid with HCl and diluted to a volume of 50 cc. to form an unknown solution for testing. 10 cc. of the unknown solution and 10 cc. of a standard iron solution containing 0.1 mg. of Fe, were tested for their color reaction with 5 cc. of a standard solution of 20 per cent potassium sulfocyanate.

- (c) With the length of the carbon chain of both dissociated and undissociated molecules.
- 2. As correlated with the polar portion of the molecule. For example,
 - (d) With the potential of a polar group of the undissociated molecule.
 - Or
 - When the molecule is dissociated.
 - (e) With the potential and kind of anion.
 - (f) With the potential and kind of cation.
- 3. As correlated with both the polar and the non-polar portions of the molecule.

A study of the normal primary aliphatic alcohols as stimulating agents in *Balanus*, *Rana*, and *Planaria* indicated that stimulation by those alcohols is correlated with the non-polar portion of the molecule (Cole and Allison, 1930). Subsequent experiments showed the same relationship for the alcohols in *Sagartia* (Cole, 1930), *Paramecium*, and *Schilbeodes*,¹ but for the fatty acids an additional effect was evident in *Sagartia* and *Balanus*. Before investigating the effects of the fatty acids thoroughly, it was deemed advisable to study the rôle of the hydrogen ion alone in a fresh water medium where the ionic conditions are much simpler than in sea water. Accordingly experiments have been done with hydrochloric acid on the solitary catfish, or mad-tom, *Schilbeodes gyrinus* Mitchill.

Methods

This animal is especially suitable for studies on chemical stimulation because it is not easily aroused by light or mechanical vibration, and because it remains quiescent for hours, unless stimulated, even when used singly. In the latter respect it differs markedly from the gregarious *Amiurus nebulosus*, which swims incessantly when alone (Parker and Van Heusen, 1917).

The animal was held in a constant position in a rectangular celluloid dish, 70 mm. long by 55 deep by 20 mm. wide, with a capacity of 35 cc. Tap water or

¹ Unpublished results obtained by Mr. S. J. Mason on *Paramecium* and by W. H. Cole on *Schilbeodes*.

the experimental solution entered the dish at the bottom near one end and flowed out at the top of the other end, after passing through Pyrex coils 4 meters long immersed in a water bath at a temperature of $18.0 \pm 0.1^\circ\text{C}$. The flow was always in the antero-posterior direction in reference to the fish and was held constant within 5 per cent at 100 cc. per minute. The content of the dish was therefore completely changed every 21 seconds. Since the head of the fish was within 5 mm. of the inlet, the experimental solution came in contact with the head almost immediately following its entrance. The reaction of the fish consisted of a sudden twitching of the whole body. Actual swimming movements were impossible due to the confining walls of the dish and to a horizontal copper screen just above the animal. Immediately following the response the solution was turned off and the fish thoroughly rinsed by allowing tap water to flow through the dish for at least 30 seconds at 250 cc. per minute. The rate of flow was then reduced to 100 cc. and the fish allowed 20 minutes for recovery. The reaction time was measured by a stop watch to 0.1 second. Under these conditions recovery was complete as shown by reproducible results. The hydrochloric acid solutions in tap water were made up fresh for each day's experiments and the hydrogen ion concentrations, which varied from $\text{pH} = 1.82$ to 6.83, were measured with the quinhydrone electrode. The pH of the tap water used for these experiments was 8.5 ± 0.5 .

RESULTS AND DISCUSSION

From the several different individuals tested similar qualitative data were obtained. For each fish the reaction time decreased as the hydrogen ion concentration increased from near neutrality to the point where toxic effects began to appear (at $\text{pH} = 1.8$). Due to the evident change in threshold from day to day the reaction times of a single fish varied considerably. Enough tests were made, however, on different fish to demonstrate clearly that the rate of stimulation increases with the hydrogen ion concentration between the limits stated. In Table I and Fig. 1 the data from a single individual collected over a period of 3 weeks are presented. The relationship between the two variables is not like the one reported for the forward movement of *Paramecium* (Chase and Glaser, 1929-30), which decreases in rate as $[\text{H}^+]$ increases. A comparable result, however, is the absence of stimulation both in *Paramecium* and *Schilbeodes* at or near the hydrogen ion concentration of the normal medium of the animal. For the catfish there is an abrupt decrease in the rate of stimulation to zero at about $\text{pH} = 7.0$. Similarly adaptation of the catfish to new hydrogen ion concentrations occurs over a period of several hours within the non-toxic limits, just as in *Paramecium*. Since the receptors concerned

in *Paramecium* and *Schilbeodes* are unknown, it is unwise to speculate as to the exact nature of the chemical processes which initiate the stimulation. Our results are quite similar to those reported by Crozier for the earthworm (Crozier, 1917-18). Although a much wider range of $[H^+]$ was used with the catfish, the same sort of curve appears if reaction time is plotted against hydrogen ion concentration. It is apparent that the catfish is very much more sensitive to changes

TABLE I

Reaction times of *Schilbeodes* to different concentrations of hydrochloric acid. Animal No. 3. Temp. = $18.0 \pm 0.1^\circ\text{C}$. Number of reactions, at each pH, 5 or more.

pH	Mean reaction time	Rate of stimulation = 100 reaction time
	<i>sec.</i>	
1.82	4.84	20.66
1.90	4.68	21.36
2.08	4.80	20.83
2.71	5.48	18.24
3.27	6.22	16.07
3.71	5.97	16.75
4.75	6.90	14.49
5.38	6.76	14.79
5.77	6.96	14.36
6.15	7.32	13.66
6.23	7.25	13.79
6.66	8.00	12.50
6.82	7.56	13.22
6.83	8.26	12.10

in $[H^+]$ from the normal, since pH = 6.8 is stimulating while for the earthworm a pH of about 2.0 was necessary under the conditions used.

In both cases the primary stimulating agent is the hydrogen ion. Sodium chloride solutions of equivalent concentrations were not stimulating to the catfish, thus demonstrating the inefficiency of the sodium ion and the chlorine ion as stimulating agents at those concentrations. It is apparent therefore that stimulation by hydrochloric acid is correlated with the potential of the cation resulting from the dissociation of the hydrochloric acid molecule, the anion being in-

effective. Since this is true, it is expected that any acid which releases hydrogen ions in sufficient concentration will owe its effect partly, if not wholly, to those ions. In the case of the fatty acids, then, even though the relative degree of dissociation is low, an increase in the hydrogen ion concentration of the medium beyond a limiting

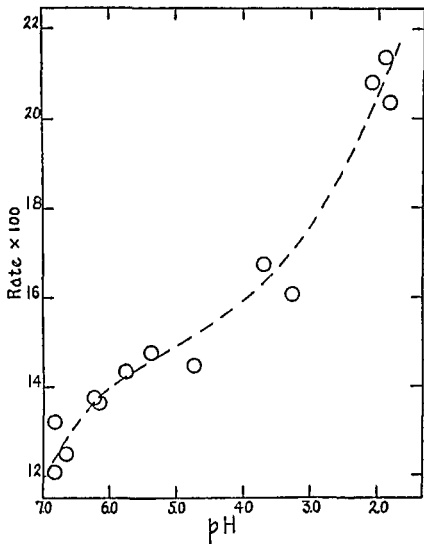


FIG. 1. Plot of the rate of stimulation $\left(= \frac{100}{\text{reaction time in seconds}} \right)$ of the catfish, *Schilbcoodes*, by hydrochloric acid at different hydrogen ion concentrations. Temp. = $18.0 \pm 0.1^\circ\text{C}$. Animal No. 3.

value would result in stimulation even though the concentration of the acid may not be sufficiently high to produce an effect through the activity of the non-polar portion of the molecule or through any of the other forces previously mentioned. Under such conditions one would expect the stimulating efficiency of the lower members

of the series to be related to the concentration of hydrogen ions produced by them. As the length of the carbon chain increases the non-polar portion of the molecule might begin to play a stimulatory rôle and with the higher members of the series it might predominate. The experiments of Crozier (1917-18) and some of our own (as yet unpublished) support this. To analyze forces such as produced by a carbon chain, it is desirable to adjust the hydrogen ion to some concentration which will not stimulate the animal. Experiments are in progress to test these possibilities.

SUMMARY

1. The reaction of the catfish, *Schilbeodes gyrimus* Mitchill, to hydrochloric acid over a wide range of concentrations (from pH 1.82 to pH 6.83) has been studied under experimental conditions which reduced to a minimum all other stimuli.

2. As the $[H^+]$ increases within the limits mentioned, the reaction time of the fish decreases. In other words, the rate of the stimulation processes is an increasing function of the hydrogen ion concentration.

3. The effective stimulus is the hydrogen ion, since NaCl solutions of equivalent concentration were not stimulating.

4. Stimulation by hydrochloric acid is therefore correlated with the potential of the cation resulting from dissociation of the acid molecule.

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THE COMBINATION OF A STANDARD GELATIN PREPARATION WITH HYDROCHLORIC ACID AND WITH SODIUM HYDROXIDE

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I

INTRODUCTION

In a recent paper the writer (1930-31b) reported a study of the isoelectric point of gelatin which had been prepared and purified according to specifications of a committee of the Leather and Gelatin Division of the American Chemical Society (Davis, Sheppard, and Briefer, 1929; Hudson and Sheppard, 1929). As a further contribution towards the work of that committee, a study has been made of the maximum combining capacity of this gelatin for the ions of hydrochloric acid, by the method used previously (1928-29) with another gelatin preparation. This led to a search for an accurate and reproducible method of drying gelatin to constant weight, and to a determination, by the usual hydrogen electrode method, of the complete titration or dissociation curve of the gelatin in solutions containing either hydrochloric acid or sodium hydroxide. While it will probably not be advisable to include the results of all these measurements in the specifications for the standard gelatin, it seems worth while to report them as a step towards the better characterization of this widely studied protein material.

II

Dry Weight Determination

In unpublished reports of the standard gelatin committee, it had been suggested that moisture be determined by drying at 105°C., either to constant weight or for

¹ Most of the experimental work was done by the writer's assistants, Miss Ruth C. Belden and Mr. Angelo E. Benaglia.

weight and the weight of solution delivered by the pipette was determined. The protein was digested over micro burners in 100 cc. Kjeldahl flasks with 2 cc. of concentrated H_2SO_4 , 1 gm. of K_2SO_4 , 0.075 gm. of HgO , and a piece of broken alundum to reduce bumping. Digestion was complete in 40 minutes, or about 20 minutes after the mixture became clear. After cooling, 35 cc. of water, 2.5 cc. of 4 per cent potassium sulfide solution, and 5 cc. of saturated NaOH were added, and the ammonia was distilled into 25 cc. of 0.02 N HCl through 2 Kjeldahl traps fused in series, as suggested by Northrop (1929-30). It was usually possible to distill over 25 cc. in 15 minutes before bumping became violent. The excess acid was titrated back with 0.02 N NaOH , using methyl red as indicator. Duplicate determinations generally agreed within 0.1 cc., or 1 part in 200, since the NH_3 was equivalent to about 20 cc. of 0.02 N HCl . Blank determinations ran from 0.04 to 0.08 cc., duplicates agreeing within 0.02 cc. By averaging four parallel determinations, rejecting any figure differing by more than 0.2 cc. from the mean of the others, the method is probably reproducible to within 2 parts in 1000. The figure obtained, from twelve determinations, for the nitrogen content of this gelatin was 18.15 per cent on the dry basis.

The specific conductivity of solutions of this gelatin at 30°C . was 3.8×10^{-5} reciprocal ohms for a 5.6 per cent solution and 5.0×10^{-5} for a 9.35 per cent solution. These figures have not been corrected for the conductivity of the water, which was about 0.2×10^{-5} .

The determination of the isoelectric point of this gelatin has been reported in a previous paper (1930-31*b*). The isoelectric point is probably at $\text{pH } 4.85 \pm 0.01$, although the method of cataphoresis of collodion particles coated with gelatin gave the value 4.80 ± 0.01 .

IV

Combination with Hydrogen and Chloride Ions from Hydrochloric Acid

This problem was studied by the method used previously with another gelatin preparation (1928-29) and with edestin (1930-31*a*). This consists in measuring the electromotive force, at 30°C ., of cells without liquid junction, of the type $\text{Ag}, \text{AgCl}, \text{HCl} + \text{protein}, \text{H}_2$. The solutions were made up by weight to contain known proportions of gelatin, hydrochloric acid, and water. The hydrochloric acid concentration was kept equal to 0.1 M while the gelatin concentrations were varied. The technique was improved by referring the gelatin concentrations to the dry basis already discussed, and by using freshly plated silver-silver chloride electrodes for each new solution. The experiments were done several times by different workers before the need for these improvements was realized, and only the latest set of

measurements is given in Table I. While the data in this table may appear rather scanty, the results of the many earlier measurements agreed with them well enough so that little doubt is held as to their reliability. Each figure for the E.M.F. is the mean of values obtained with three separate cells, all agreeing to within 0.0002 volt, and each constant to 0.0001 volt for at least 1 hour. Similar reproducibility and constancy were attained on refilling the cells with fresh solution and taking readings for another hour after the electrodes had reached equilibrium. The experimental data are given in the first three columns of Table I.

TABLE I

Electromotive Force at 30°C. of the Cells Ag, AgCl, HCl + Gelatin, H₂

<i>m</i>	<i>g</i>	<i>E</i> (observed)	<i>E'</i> ₀	<i>E</i> (calculated)	ΔE	pH (approx.)
0.1000	50.3	0.3708	0.2306	0.3709	+0.0001	1.3
0.1000	70.4	0.3841	0.2304	0.3840	-0.0001	1.5
0.1000	90.5	0.4084	0.2302	0.4084	0	1.9

m = mols HCl per 1000 gm. H₂O.

g = gm. dry gelatin per 1000 gm. H₂O.

E (observed) = E.M.F. in volts, corrected to 1 atmosphere dry H₂.

*E'*₀ = *E* + 0.1203 log *m*, for cells containing HCl alone, of molality equal to *m* - *gy* (*E'*₀ = 0.2310 for *m* = 0.1002).

E (calculated) = *E'*₀ - 0.06015 log (*m* - *gx*) (*m* - *gy*).

x = mols H⁺ combined with 1 gm. gelatin = 9.58 × 10⁻⁴.

y = mols Cl⁻ combined with 1 gm. gelatin = 2.0 × 10⁻⁴.

ΔE = *E* (calculated) - *E* (observed).

pH (approx.) = - log (*m* - *gx*).

The interpretation of these data depends on the following considerations. The E.M.F. of a cell such as those measured must be given by the thermodynamic equation

$$E = E_0 - 0.06015 \log m_{\text{H}} m_{\text{Cl}} \gamma^2 \quad (1)$$

which holds for 30°C. Here *E* is the observed E.M.F. in volts, after correction to unit pressure of hydrogen, *E*₀ is a constant depending on the nature of the electrodes and the temperature, *m*_H and *m*_{Cl} are the molalities of free H⁺ and Cl⁻ in the solution bathing the electrodes,

and γ is the geometric mean activity coefficient of the ions of HCl in the solution.

If some of the ions from the added HCl are combined with gelatin, it follows that $m_H = m - gx$ and $m_{Cl} = m - gy$, where m is the molality of the total HCl, free and combined, g is the gelatin concentration in gm. per 1000 gm. H_2O , and x and y are the numbers of mols of H^+ and Cl^- , respectively, combined with 1 gm. of gelatin. Equation (1) may be made more useful by these substitutions, and by letting $E'_0 = E_0 - 0.1203 \log \gamma$. It then becomes

$$E = E'_0 - 0.06015 \log (m - gx) (m - gy). \quad (2)$$

In order to solve equations of this form for x and y , it is necessary to have values for E'_0 . This quantity includes E_0 , which should not be changed by the presence of protein, and γ , which may well be changed by it. As a first approximation it was assumed that γ , and hence E'_0 , was unchanged by the gelatin. Using for E'_0 the value of 0.2310, obtained from measurements with HCl free from protein, of molality 0.1002, the three equations resulting from the three experiments of Table I were represented graphically by assuming values for x , calculating y , and plotting y against x . The curves so obtained intersected at points corresponding to $x = 9.6$ to 9.7×10^{-4} and $y = 1.6$ to 1.8×10^{-4} . As a second approximation⁴ it was assumed that E'_0 should be equal to the value obtained with pure HCl, not of molality m , but of molality $m - gy$. The reason for this is that gy represents a part of the HCl of which not only the H^+ but also the Cl^- is bound to gelatin; hence this part should have as little influence on the activity coefficient as a non-electrolyte. The difference $m - gy$ represents some free HCl and some HCl of which only the H^+ is bound to gelatin. If the ionic strength principle of Lewis and Randall (1921) applies to mixtures of HCl and an ionized protein hydrochloride, and if the positive protein-hydrogen ion is assumed to have an effective valence of one in its effect on the ionic strength, then the value of γ or E'_0 for

⁴ This method of approximation, which is the same as that used in the previous study of edestin, was suggested by Dr. Rubert S. Anderson. The writer wishes to acknowledge his indebtedness to Dr. Anderson for much helpful discussion as well as for some careful experiments which were carried out in the preliminary stages of the work.

such a mixture should be the same as that for HCl of molality $m - gy$. For the second approximation y was assumed to be 1.7×10^{-4} , and values of E'_0 were read off from a plot of the values obtained for HCl in connection with the study of edestin (1930-31a), E'_0 being plotted against \sqrt{m} . These values were raised by 0.0001 volt because the value for 0.1 M HCl with the new electrodes was 0.2310 instead of 0.2309, as in the previous work. A graphical solution of the equations on this basis gave curves intersecting at points whose coordinates averaged 9.6 and 2.0×10^{-4} . A third approximation on the basis of $y = 2.0 \times 10^{-4}$ gave intersections for which the average value of y was again 2.0×10^{-4} , indicating that no further approximations were possible. The values of E'_0 corresponding to $m - gy$, using this value for y , are given in the fourth column of Table I. The fifth column gives values of E calculated from equation (2) by the use of these values for E'_0 and the values $x = 9.58 \times 10^{-4}$ and $y = 2.0 \times 10^{-4}$. The sixth column shows the extent to which the experimental data are fitted by these values. The last column gives the negative logarithm of the molality of hydrogen ion, which is approximately the pH value of the solutions. Table I shows that the data may be explained by assuming that each gm. of this gelatin in 0.1 M HCl was combined with 9.58×10^{-4} mols of H^+ and 2.0×10^{-4} mols of Cl^- , these amounts being independent of the gelatin concentration and hence of the pH, within the limits given.

The same data were treated in another way by solving equation (2) for three unknowns, E'_0 , x , and y , using again the experimental figures in the first three columns of Table I. The resulting values were $E'_0 = 0.2307$, $x = 9.55 \times 10^{-4}$, and $y = 2.03 \times 10^{-4}$. From these values it is possible to get exact agreement between the observed and calculated values of E . This agreement, however, is a necessary consequence of the fact that only three equations were solved in getting the three unknowns. Theoretically E'_0 should not be constant, as already explained, and if it were constant it might be expected to have the value 0.2310 which was used in the first approximate calculation of x and y . Yet the agreement of the values for x and y found in this way with those given in Table I is an indication that the values found for x and y may be, to a certain extent, independent of the assumptions used in calculating them.

V

Combination Curve from pH Measurements

As a further means of characterizing this standard gelatin preparation, experiments were carried out to determine its complete dissociation curve, or curve of combination with H^+ and OH^- . This curve was obtained by making up a series of solutions of approximately constant gelatin concentration with varied concentrations of HCl or NaOH, rather than by the titration of a single gelatin solution. Solutions were made up by weight, as before, and concentrations were referred to 1000 gm. of water. The pH determinations were made at $30^\circ C.$ with hydrogen electrodes in the vessels described by Simms (1923), liquid junction being made with saturated KCl in an open stop-cock. The standard of pH was 1.075 for 0.1000 molal HCl, possible variations in liquid junction potentials being neglected. Instead of plotting the total concentration of acid or alkali directly against pH, which would give an experimental titration curve, a calculation was made to get the number of equivalents of H^+ or OH^- combined with each gm. of gelatin. The curve of this quantity against pH is the dissociation or combination curve of the protein. The calculation was made by the method previously used (1928-29), which is essentially similar to that of Cohn (1925).

In the case of solutions containing HCl, the quantity plotted was $\frac{b_H}{g}$ where b_H is given by the relation

$$b_H = m - \frac{a_H}{\gamma_H}. \quad (3)$$

Here g is the gelatin concentration in gm. per 1000 gm. H_2O , b_H is the molality of combined H^+ , m the total molality of HCl, and a_H the activity of H^+ as obtained from the pH measurements by the relation

$$pH = -\log a_H. \quad (4)$$

The quantity γ_H is the activity coefficient of the hydrogen ion. This was obtained from measurements of pH of HCl solutions without protein, the assumption being made that γ_H for a given value of m was not altered by the presence of protein. The values of γ_H so

obtained were practically identical with those given by Scatchard (1925) for 25°, as might be expected, since the standard of pH used in this work was obtained by arbitrarily assigning to 0.1 M HCl at 30° the value of γ_H which Scatchard found for 25°.

In the case of NaOH solutions, the quantity plotted was obtained by a similar equation,

$$b_{OH} = m - \frac{a_{OH}}{\gamma_{OH}}. \quad (5)$$

Here m is the total molality of NaOH, b_{OH} the molality of combined OH^- , a_{OH} the activity of free OH^- , and γ_{OH} its activity coefficient. The values of a_{OH} and γ_{OH} were obtained from pH measurements by the following method, which is believed to be new. Measurements were made of the pH of a series of NaOH solutions without protein, the values being referred to the HCl standard already mentioned. The pH values for NaOH solutions were not so reproducible as in the case of HCl, the divergence of duplicate solutions prepared and measured at different times being in some cases as much as 0.03 pH. By averaging four or five measurements at each concentration, fairly reliable values were obtained for 0.02, 0.04, 0.05, and 0.1 M. From these average pH values, values of the quantity $pH - \log m$ were calculated and plotted against \sqrt{m} . The four points fell within 0.002 pH of a straight line whose equation was

$$pH - \log m = 13.724 - 0.4 \sqrt{m}. \quad (6)$$

By extrapolating this line to zero concentration, it was possible to calculate values of the activity coefficient of OH^- for any solution of NaOH of molality up to 0.1. From the relations

$$a_H a_{OH} = K_w \quad (7)$$

and

$$a_{OH} = m \gamma_{OH} \quad (8)$$

it follows that, for pure NaOH solutions,

$$a_H = \frac{K_w}{m \gamma_{OH}} \quad (9)$$

or

$$pH - \log m = pK_w + \log \gamma_{OH}. \quad (10)$$

From the theoretical equation (10) and the empirical equation (6) it appears that pK_w corresponds to 13.724 and $\log \gamma_{OH}$ to $-0.4 \sqrt{m}$. It may be noted that Michaelis (1922) gives 13.725 as the value of pK_w at 30° , while the limiting law of the Debye-Hückel theory states that $\log \gamma$ for a uni-univalent electrolyte should approach $-0.5 \sqrt{m}$ in very dilute solutions.

Here use was made only of the empirical relation

$$pK_w + \log \gamma_{OH} = 13.724 - 0.4 \sqrt{m} \quad (11)$$

as a means of getting values of $\frac{K_w}{\gamma_{OH}}$ for use in equation (12) with the pH measurements of the gelatin solutions containing NaOH. Equations (5) and (7) give

$$b_{OH} = m - \frac{K_w}{a_H \gamma_{OH}} \quad (12)$$

in which m is the total molality of the NaOH in the gelatin solution, a_H is obtained from the pH data by equation (4), and the ratio $\frac{K_w}{\gamma_{OH}}$ is obtained from equation (11). Finally the values of b_{OH} obtained by equation (12) were divided by g , the gelatin concentration, to get the number of mols of OH^- combined with 1 gm. of gelatin. The results of some of the measurements and calculations are given in Table II, and the complete dissociation curve of the gelatin is given in Fig. 2, which shows the amounts of combined H^+ or OH^- per gm. of gelatin as a function of pH.

It may be seen from Fig. 2 that on the acid side the amount of H^+ bound per gm. gelatin reached a limiting value of 9.6×10^{-4} equivalents, which is in good agreement with the value 9.58 found by the more precise method of Section IV. The curve crosses the line of zero combination at pH 4.85, which is the value found for the isoelectric point of this gelatin in the previous study (1930-31*b*). On the alkaline side the curve shows a flattening near pH 8, indicating that one set of groups is completely neutralized in this region. This is in agreement with the titration curves of Loeb (1922) and the writer (1923-24), as recalculated by Cohn (1925). The amount of bound OH^- at this

TABLE II

Combination of Gelatin with H^+ from HCl and OH^- from $NaOH$ as Calculated from pH Measurements at $30^\circ C$.

A. Gelatin + HCl

m	g	pH	$-\log \gamma_H$	m_H	b_H	$\frac{10^4 b_H}{g}$
0.1022	20.4	1.157	0.075	0.0828	0.0194	9.51
0.0510	20.35	1.566	0.064	0.0315	0.0195	9.58
0.0306	20.3	2.015	0.056	0.0110	0.0196	9.65
0.01993	19.95	2.678	0.049	0.00235	0.01758	8.82
0.01529	20.3	3.230	0.046	0.00066	0.01463	7.21
0.00996	19.9	3.714	0.039	0.00021	0.00975	4.89
0.00498	19.9	4.188	0.031	0.00007	0.00491	2.46
0.00199	19.9	4.544	0.021	0.00003	0.00196	0.98
0	120.0	4.86	0	0.00001	0	0

B. Gelatin + $NaOH$

m	g	pH	$pK_w + \log \gamma_{OH}$	m_{OH}	b_{OH}	$\frac{10^4 b_{OH}}{g}$
0.00200	20.0	5.220	13.707	$10^{-6.8}$	0.00200	1.00
0.00500	20.0	6.000	13.696	$10^{-7.7}$	0.00500	2.50
0.00718	20.2	7.659	13.690	$10^{-8.0}$	0.00718	3.55
0.00821	20.2	8.763	13.689	0.00001	0.00820	3.73
0.01000	20.0	9.624	13.684	0.00009	0.00991	4.96
0.01235	20.1	10.155	13.680	0.00030	0.01205	6.00
0.01547	20.1	10.651	13.675	0.00095	0.01452	7.22
0.02000	20.0	11.219	13.668	0.00356	0.01644	8.23
0.0474	31.1	11.932	13.637	0.0197	0.0277	8.91
0.0974	41.4	12.361	13.599	0.0578	0.0396	9.57

m = molality of HCl or $NaOH$.

g = gm. gelatin per 1000 gm. H_2O .

$pH = 1.075 + \frac{E - E_{HCl}}{0.06015}$, where E = E.M.F. observed with calomel cell against

H_2 electrode in gelatin solution, and E_{HCl} = E.M.F. observed with the same calomel cell against H_2 electrode in 0.1000 M HCl .

$-\log \gamma_H$ was obtained from a plot of $pH + \log m$ against \sqrt{m} , using data for HCl without protein.

m_H was obtained from the relation $-\log m^H = pH + \log \gamma$.

$b_H = m - m_H$.

$pK_w + \log \gamma_{OH}$ was obtained from a plot of $pH - \log m$ against \sqrt{m} , using data for $NaOH$ without protein.

m_{OH} was obtained from the relation $pK_w + \log \gamma_{OH} - pH = -\log m_{OH}$.

$b_{OH} = m - m_{OH}$.

point was somewhat higher for the standard gelatin than for the preparations studied earlier, being about 3.6×10^{-4} equivalents per gm. This figure was roughly confirmed by a colorimetric titration with phenol red as indicator, which gave an end point of 3.3×10^{-4} equivalents NaOH per gm. gelatin at pH 7.9.

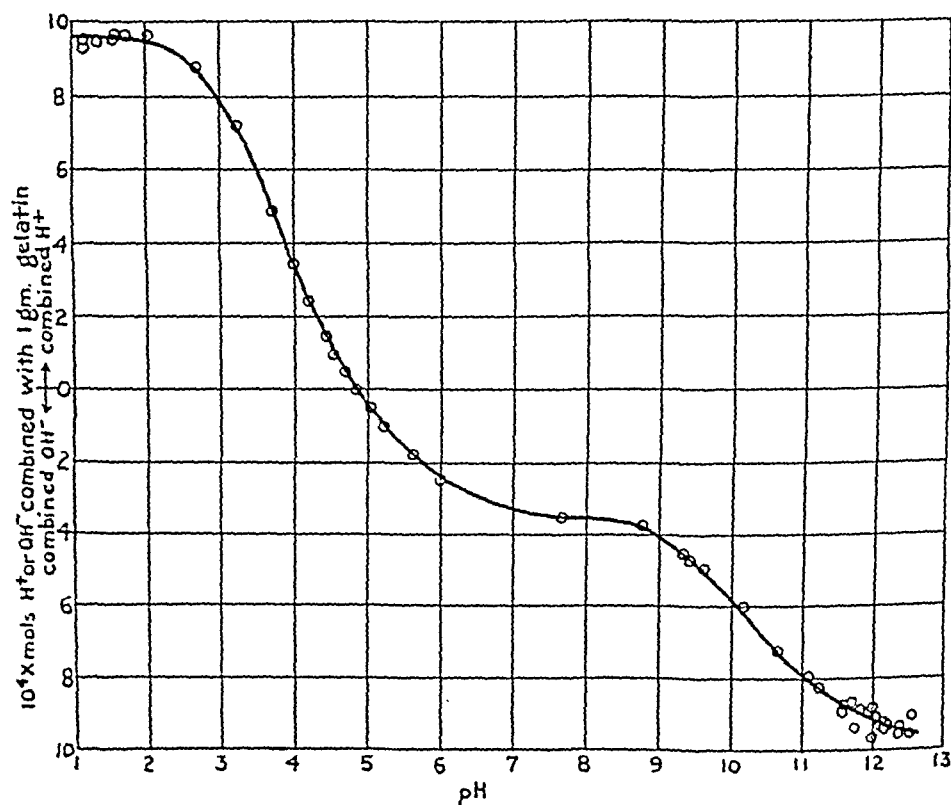


FIG. 2. Combination curve of the standard gelatin preparation with H^+ from HCl and OH^- from NaOH, as obtained from pH measurements at $30^\circ C$. The abscissae are pH values; the ordinates are $10^4 \times$ mols of H^+ or OH^- combined with 1 gm. of gelatin.

The remainder of the curve, in the more alkaline region, is quite different from Cohn's curve of collected data on various gelatins, in that it shows no flattening in the vicinity of 6×10^{-4} equivalents of combined OH^- . The scattering of the points near pH 12 is such that it cannot be said definitely that a maximum combining capacity for OH^- is indicated. At any rate it seems certain that this gelatin, or

its products of decomposition, combined with as much as 9 or 9.5×10^{-4} equivalents of OH^- between pH 12 and 12.5. This high combining capacity seems to have been characteristic of this particular lot of gelatin (Eastman Standard Gelatin, Lot 48). Another lot purchased later from the same source (Eastman Purified Gelatin, Lot 51) gave a combination curve practically identical with that here published, except that it flattened off at about 8.4×10^{-4} equivalents of combined OH^- per gm. between pH 11.5 and 12.5. Apparently it may be concluded either that the preparation of gelatin is in need of further standardization, or else that the maximum combining capacity for alkali is a property not well suited to the exact characterization of gelatin preparations.

VI

SUMMARY

It has been found possible to obtain constant dry weights of sheet gelatin only by drying *in vacuo* at temperatures below 100°C . for a period of several weeks. Values are given for the ash and nitrogen content, the specific conductivity, and the isoelectric point of a standard gelatin preparation. By the method of E.M.F. measurements of cells without liquid junction, of the type $\text{Ag}, \text{AgCl}, \text{HCl} + \text{gelatin}, \text{H}_2$, it has been found that this gelatin in 0.1 M HCl combines with a maximum of 9.58×10^{-4} equivalents of H^+ and 2.0×10^{-4} equivalents of Cl^- . By means of pH measurements with the hydrogen electrode and a KCl junction, the combination curve of this gelatin with H^+ from HCl and OH^- from NaOH has been determined between pH 1.1 and 12.5.

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PHOTOCELL ANALYSIS OF THE LIGHT OF THE CUBAN ELATERID BEETLE, PYROPHORUS

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(Accepted for publication, June 26, 1931)

The light of *Pyrophorus* comes from two dorsal prothoracic organs which are very conspicuous, resembling automobile headlights, and a single median organ on the anterior ventral region of the abdomen, only visible when the abdomen is bent upward during flight. The anatomical and physiological features have been studied in great detail by Heinemann (1872 and 1886) and Dubois (1886) and the results published in extensive monographs.

The prothoracic organs are easily accessible without injury or drastic disturbance of the insect, and it is the light from these which has been recorded and described in this paper. When mechanically disturbed, as during handling or gently squeezing between the fingers, these organs glow brilliantly for a considerable time, the duration depending on the individual and the amount of disturbance. Unlike the lampyrid fireflies, the light lasts long enough to make comparisons of its brightness with a standard, and Harvey and Stevens (1928), using a modified Macbeth illuminometer, found a maximum value of 0.045 lamberts among twenty-five specimens.

The luminescence rises quickly to a maximum and then appears quite steady to the eye, sometimes varied with an occasional fluctuation in brightness, if squeezed more forcibly. After the light has nearly subsided, rhythmic fluctuations in intensity may be observed with the eye occurring at intervals of 0.8 to 2 seconds. This rhythmic fluctuation can sometimes also be observed when the animals are walking around in a dish in a dark room. In order to analyze the rate of development of light, which might show some points of interest, the beetles were arranged in front of a large photoelectric cell connected with an amplifier and a string galvanometer. The system was the one

used by and fully described by Harvey and Snell (1931) for recording rapid flashes of luminescence on mixing *Cypridina* luciferin and luciferase solutions, and by Snell (1931) for recording the normal flash of the firefly, *Photuris pennsylvanica*, which lasts only $\frac{1}{8}$ to $\frac{1}{10}$ second.

The rate of development of luminescence shows nothing unusual, but many records exhibit a small rhythmic change in light intensity which is quite imperceptible to the eye and could only be detected by this method of analysis. The final method of holding the beetles before the photocell was as follows: A test-tube about 25 mm. diameter, its rounded end directed toward the sensitive surface, is clamped before the photocell on a stand supported from the wall of the room and not connected in any way with the table on which the photocell and amplifier stand. The separate clamping system is necessary because of the extreme sensitivity of the photocell amplifier system to vibration, despite the fact that it is protected by sponge rubber cushions. The beetle is placed in the rounded end of the test-tube, dorsal side toward the photocell, and held in position by a plug of cotton lightly pressed against the insect. To excite luminescence, it is only necessary to press firmly against the cotton with an ebonite rod. The glow begins immediately and may last several minutes. On humid days this scheme worked perfectly but on dry days several records were spoiled by electrification of the glass tube when the cotton was rubbed over it. The static charge caused marked deflections of the string. They were eliminated by moistening the cotton.

Fig. 1 shows several typical curves. Only the beginning can be given, as the light is apt to fade out gradually and at an unpredictable time. It will be observed that 0.8 to 1 second is taken for maximum brightness to be attained and that the rate of increase in brightness is more or less linear over the greater part of its course, only the beginning and end showing deviations. If the records were shortened along the time axis the curve of development of luminescence would be straighter, but somewhat resembling the flash of a firefly, except that the firefly takes only 0.06 second to develop its maximum light.

The striking phenomenon (*B* and *D*) is the rhythmic change in light intensity, which sometimes starts at a rate of 300 per minute (period 0.2 seconds), but more frequently shows a rate of 214 per minute (period 0.28 second) falling off after 20 seconds to 150 a minute (period of

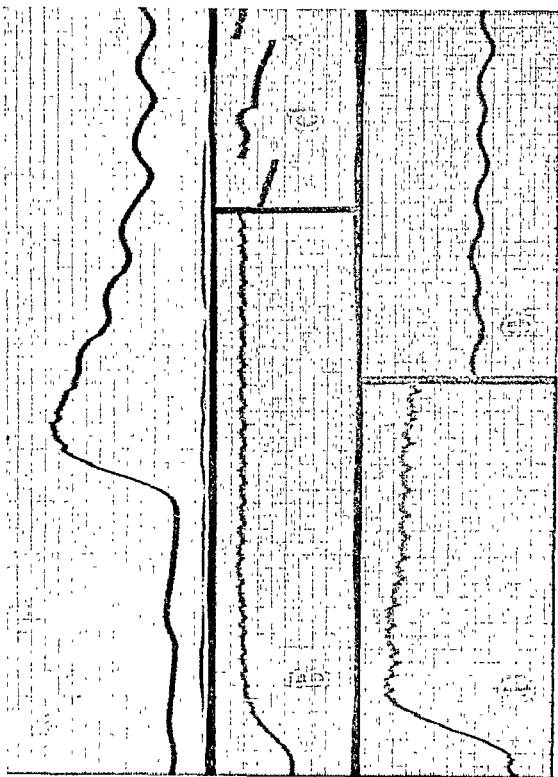


FIG. 1. *A.* Large *Pyrophorus* (32 mm.) showing slow rhythmic pulses of prothoracic light organs and the increase in brightness on stimulation, which falls off into more rapid rhythmic pulses of light. Time 0.2 second. *B.* Small *Pyrophorus* (22 mm.) showing rapid rhythmic changes in luminescence. Time 0.2 second. *C.* Large *Pyrophorus* (34 mm.) held before photocell by hand showing effect of click reflex in changing position of light organs before photocell. Note sudden increase in light striking cell as each click (C_1 and C_2) brings luminous organs nearer the photocell. Time 0.1 second. *D.* Another small *Pyrophorus* (24 mm.) showing rapid rhythmic variations in light intensity which gradually change after 15 seconds (omitted from record) to D_1 , rhythmic pulsing of light in the organs

0.34 to 0.4 second). The No. 5 record of May 27, 1931, gave an average period 0.4 second (150 per minute) for the first 20 seconds with a rhythm actually spaced as follows:—30, 29, 32, 34, 40, 32, 36, 40, 38, 28, 32, 29, 52, 34, 38, 42, 52, 52, 46, 44, 36, 32, 44, 46, 36, 32, 48, 36, 40, 42, 42, 44, 48, 44, 32, 44, 44, 36, 52, 38, 40, 48, 40, 29, 44, 48, 36, 44 hundredths of a second.

The change in light intensity during this rhythm only amounts to 5 to 6 per cent of the maximum intensity, so that it is not surprising to find the effect undetectable by eye. The period of the string is approximately 0.008 second at the tension used, so that more rapidly occurring rhythms could easily be detected, even though persistence of vision would cause fusion to the eye. To make sure that the *Pyrophorus* light is not made up of individual flashes of even more rapid rhythm, a beetle was fixed on a centrifuge head of 26 cm. diameter and observed in a dark room while the speed of revolution was increased gradually up to 3000 R.P.M. At no speed did the luminescence appear anything but continuous. Since the animal travelled 40,000 mm. per second at the highest speed, a rhythmic flashing of 40,000 per second or less should have been easily detectable. We may conclude that the light of *Pyrophorus* is truly continuous (except for the rhythm displayed in the records) although it must be borne in mind that if the individual cells flashed intermittently at a rapid rate and were out of phase, the integrated result would be a continuous light.

The question arises as to the cause of the rhythm of 300 to 150 per minute. We may state at the start that this luminescence rhythm is no artifact due to vibration or joggling of the apparatus. It has appeared in records of four individuals taken on different days and under conditions where joggling was impossible. It has not, however, appeared in every record. An especially large *Pyrophorus* (34 mm. long) on squeezing emitted light of relatively short duration which showed no small rhythmic changes but only fluctuations in intensity which broke into the slow marked variations in luminescence of about one second period.

There are four possible explanations of the rapid rhythm. It may be connected with the (1) click reflex, (2) respirations, (3) heart beat, or (4) rhythmic nerve discharges to the organ.

1. These beetles, members of the Elateridae, exhibit the click reflex,

a sudden powerful movement of the thorax by which they project themselves into the air when lying on their back. When squeezed between the fingers they click rhythmically at a rate of about one per second. The change in position of the thorax during this movement might change the position of the light organs with reference to the photocell sufficiently to affect the record. This cannot be the explanation of the rhythm in the record because the rhythmic variations in light intensity are too rapid and the curve of each variation would be different, a sudden change in light intensity followed by a slow recovery, as can be proven by actual records of the light while clicking is occurring. Fig. 1-C is such a record showing two clicks. I have also noticed no indication of clicking when the beetles are pressed by cotton against the end of the test-tube.

2. The respiratory rhythm might force more air into the organ. Indeed, Heinemann has observed a rhythmic luminescence of the abdominal organ synchronous with breathing movements. He also believes that stimulation of the nerve cord causes luminescence by a secondary effect, the stimulation of the respiratory muscles which then force air into the organs. However, the rhythm of breathing is much slower than the light rhythm exhibited in the records. After removal of the elytra and wings I have observed a maximum respiratory rate of fifteen per minute. Dubois, who believes respiration has no direct influence on luminescence, recorded graphically the breathing movements of *Pyrophorus*. His records show 10, 20, and 24 respirations per minute and in addition *the cessation of respiration when the prothoracic organs light*. The rapid rhythm in my records cannot be due to respiration. Even the slow marked rhythmic variations in light intensity which have a period of 0.8 second (75 per minute) to 2 seconds are fast for a respiratory rhythm, but it is quite conceivable that they may be caused by some muscular mechanism connected with local distribution of air in the tracheae of the thorax. These pulses of light occur at the end of a period of lighting and are easily detectable by eye since they represent a 25 per cent or more change in light intensity. Fig. 1, A and D₁, are records of this slower "pulsing" of the light organ.

3. It is very unlikely, from the physiological scheme in insects, that heart beat should influence luminescence intensity, since oxygen is carried to the light organ by tracheae directly and not through the

blood. In addition even rate of heart beat is slower than the rhythm of luminescence. Dubois states that the heart rate is 106 per min. after removing some tergites to observe the dorsal vessel, and subsequently drops to 60–70. The rate increases from 72 to 84 when the animal is excited so that its prothoracic organs light. I have observed a rate of 84 per min. at 28° C. after removing the elytra and wings but leaving the tergites uncut. Apart from the improbability of the heart rate affecting luminescence these rates are all too slow to account for the rhythm during the bright luminescence of a prothoracic organ. However, the heart beat is of a proper rate to agree with some of the marked fluctuations of intensity after the bright luminescence is over, but I believe this relation is pure coincidence.

4. We are forced to fall back on the most likely explanation, namely a rhythmic discharge from the nerve centre for the organs, a volley similar to that sent out by the respiratory centre of mammals. Records *B* and *D* of Fig. 1 look very much like an incomplete tetanus of muscle and may be attributed to the same cause, rhythmic stimulation. Whether this is a direct stimulation of photogenic cells or an indirect one, operating through a mechanism closing and opening the tracheal ends, cannot be stated at the present time. According to Dubois the light organ of *Pyrophorus* lacks tracheal end-cells, so that a direct stimulation to luminescence seems most likely.

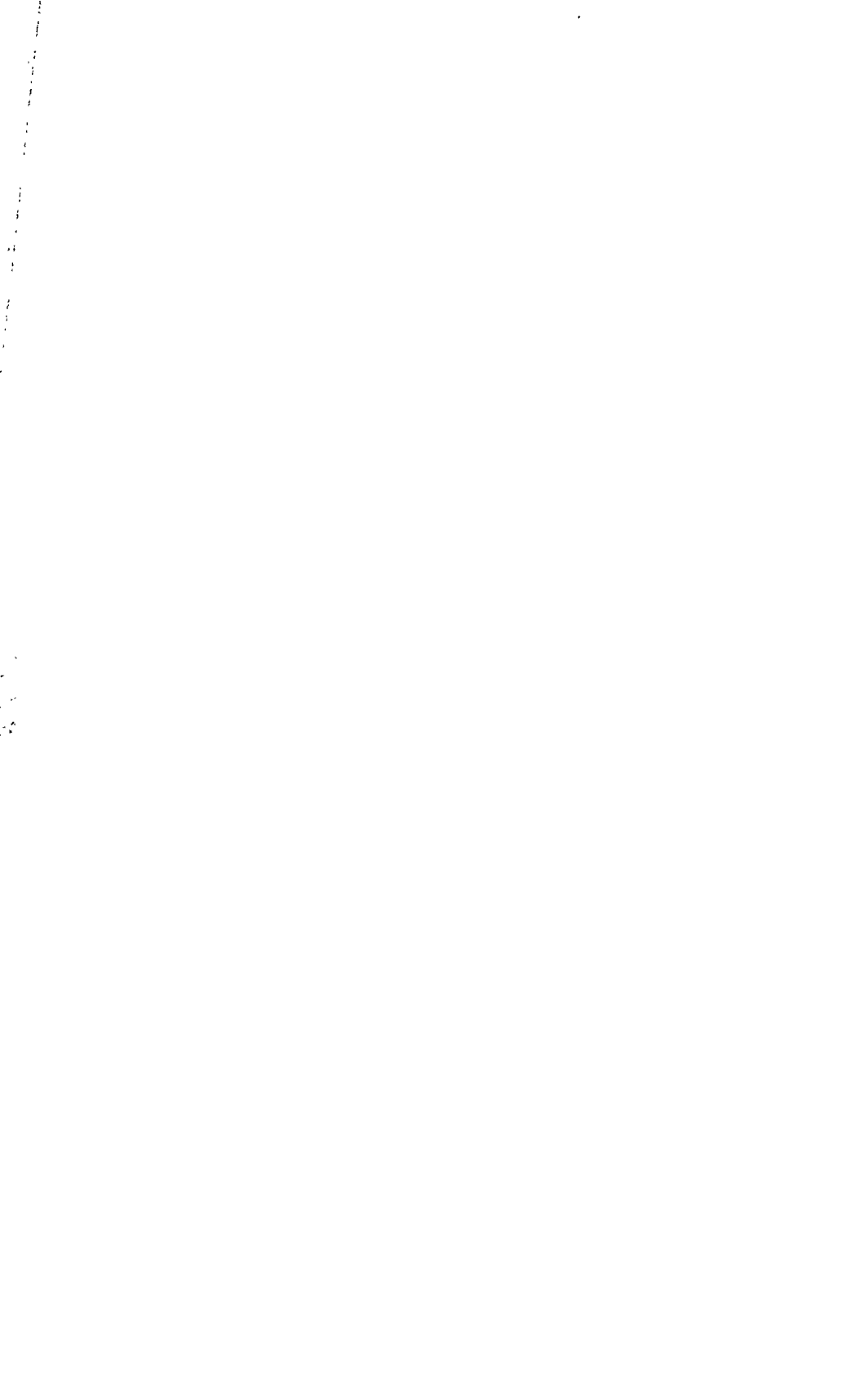
SUMMARY

Records are reproduced showing various types of luminescence intensity—time curves for the prothoracic light organs of *Pyrophorus*. A small (5 per cent) rhythmic fluctuation of light intensity during the reflex emission of light is to be observed in many records, which because of its rapid rate is attributed to rhythmic nerve discharge from a photogenic centre. Longer “pulsations” of luminescence intensity (25 per cent change) can be detected by the eye.

In conclusion I wish to thank my assistant, Mr. Charles Butt, for his aid in taking the records.

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HIGH SPEED PHOTOMICROGRAPHY OF LIVING CELLS SUBJECTED TO SUPERSONIC VIBRATIONS

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(Accepted for publication, August 25, 1931)

The destruction of cells by high frequency sound waves (supersonics or ultrasonics) is undoubtedly connected with the cavitation (separation) of dissolved gas from the liquid phase, as has been shown by Johnson (1929) and confirmed by Schmitt and Uhlemeyer (1930) and by Harvey (1930). No cavitation and no destruction of cells occurs with low tension of dissolved gas or if the hydrostatic pressure on the fluid is increased without dissolving more gas. It seems most likely that minute cavitated gas bubbles *outside* the cell are responsible for the effect. This is indicated by the fact that plant cells are destroyed in water saturated with air despite the fact that the hydrostatic turgor pressure within these cells may be 4 or 5 atmospheres (Harvey, 1930).

The question arises whether high speed instantaneous photographs may not aid in the analysis of the destruction. With commercial moving picture cameras it is a simple matter to photograph cells at speeds of 128 pictures a second. It is only necessary to support the camera (f. 1.8 lens), focused for infinity, above the microscope and use a sufficiently bright source of illumination. Perfectly exposed pictures can be obtained with the image of a Pointolite lamp focused on the material for low powers (100 magnification), or by use of an image of the sun (for a few seconds) for higher powers.

In this way moving pictures of ciliary motion, and sea urchin eggs subjected to supersonic waves have been obtained. These pictures showed that sea urchin eggs may be completely cytolysed in one frame (1/128 second) (Harvey, 1930). To analyse the cytolysis by supersound, higher speeds were necessary and the following type of camera was constructed, capable of taking 1200 or more pictures per second.

On one end of the shaft of a small variable speed motor, Mo, (Fig. 1) is placed a right angle prism (P) and on the other end a bakelite disk (D) (5 inches diameter) having 30 brass contact surfaces (about $3/32$ inch diameter) embedded in it. The prism projects into a wooden box (B) which serves as a camera box and is attached to the frame of the motor. Its lower surface contains an opening which can be covered. It can be loaded with a strip of movie film, F, which is fastened about the circumference of a circle whose diameter is 10 inches. The motor and camera box are mounted on a firm support immediately over a $\times 20$ microscope objective (O), focussed on the material to be studied, placed on top of a polished quartz crystal (C) with tin foil electrodes (E). The electrodes are connected to an oscil-

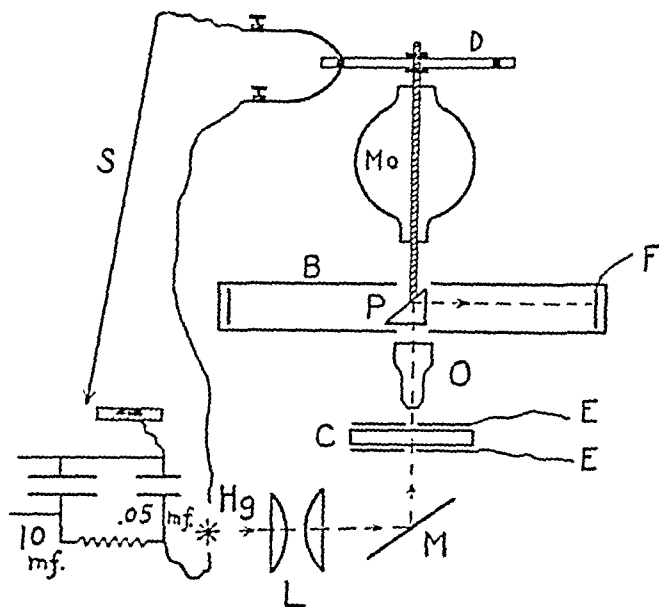


FIG. 1. Diagram of camera and lighting system for high speed photography of living cells. Mo, motor; D, disk with thirty contacts; B, camera box; F, film; P, total reflecting prism; O, $\times 20$ objective; C, quartz plate; E, electrodes; M, mirror; L, lens; Hg, lamp; S, pendulum swinging across contact.

later tuned to 415 kilocycles, the natural period of the quartz crystal. A thick cover glass cemented to a glass rod, not shown in the diagram, movable vertically by a micro manipulator allows the thickness of sea water containing eggs to be adjusted at will. Fig. 2 is a photograph of the set-up.

If a bright light is flashed on for a few millionths of a second, with every contact of a revolving disk 30 pictures will be recorded on the film and the rate at which pictures are recorded will depend on the rate of revolutions of the disc. We have usually run at a speed of 40 r.p.s. which makes 1200 pictures per second. The speed of the disk can be easily determined by pasting a strip of white paper on it

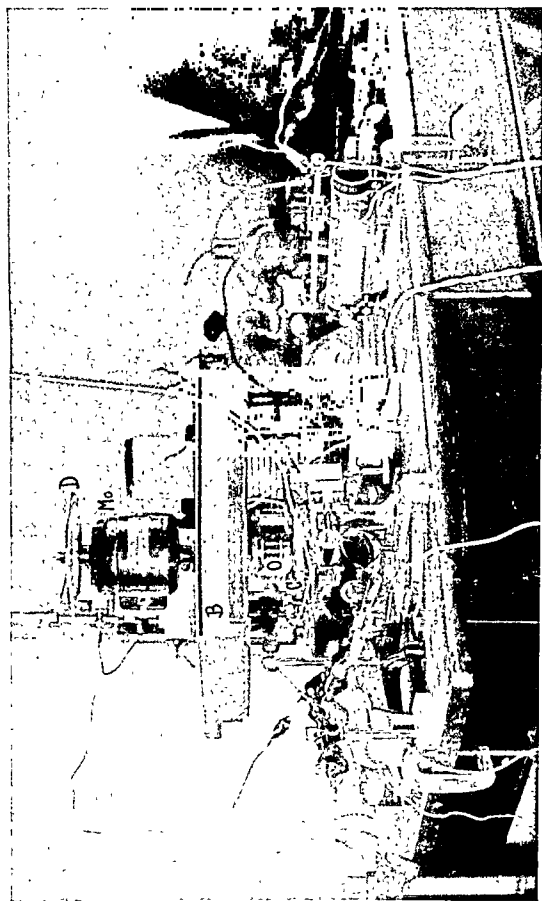


FIG. 2. Photograph of camera and lighting system exclusive of condensers and transformers with crystal oscillator in background.

and observing this during revolution with a neon lamp run on the 60 cycle A.C. lighting circuit. The lamp flashes on and off 120 times a second so that two images correspond to 60 R.P.S., three images to 40 R.P.S., etc., of the motor. The speed is then regulated with a rheostat to the desired value.

The source of illumination (Hg) is similar to the one used with the microscope-centrifuge (Harvey and Loomis, 1930; Harvey, 1931), a 4500 volt 0.05 microfarad condenser discharge through heated mercury vapor at approximately atmospheric pressure in a small Pyrex tube. The heating of the Hg vapor is carried out electrically by a coil of nichrome wire wrapped on a quartz tube enclosing the Pyrex tube. Quartz is necessary since hot glass becomes too good a conductor for the high voltages used. The 0.05 microfarad condenser is charged between contacts through a high resistance of very dilute CuSO_4 in water, from a reservoir condenser of 10 microfarad capacity, in its turn charged from 4500 volt transformers with full stage rectification, using CR 0100 mercury tubes. The light is focused on the material to be photographed by means of a lens (L) and mirror (M).

In order that pictures may not be taken for more than one revolution of the disk the lamp circuit is turned on for the proper length of time by allowing a pendulum (S) with contact point to sweep across a surface of metal whose width can be adjusted. By swinging the pendulum from various heights the time of contact can be varied to correspond to motor speed so as to take just thirty pictures. The pendulum also carries a second contact which sweeps across a second surface of metal at the same time but so adjusted as to make contact slightly after the lamp is in circuit. The negative high voltage to the plate of the oscillating tube to drive the quartz crystal passes through this contact so that the supersonic waves start a few (about six) pictures after the lighting system is on. The oscillator is on during the last 80 per cent of the time the pictures are being taken.

The supersonic oscillator was the small 75 watt outfit described by Harvey and Loomis (1928) and Harvey, Harvey, and Loomis (1928). The only change necessary was 2000 volt *direct* current to the plate of the oscillating tube from a 2000 volt motor generator with a 10 microfarad condenser across the terminals. This insures continuous vibration of the crystal instead of vibration in 60 cycle pulses as in our former set where the plate voltage was supplied by a transformer.

The image of the cells reflected on the film by the right angle prism revolving 40 R.P.S., is travelling at a rate of 32000 mm. per second or about 0.1 mm. in 3 micro-seconds. Since the photographic image taken at this speed is quite unblurred the condenser discharge must last less than a micro second and is consequently quite adequate for the purpose.

Fig. 3 shows unfertilized eggs of *Arbacia* (diameter 75 micra) in process of disintegration photographed at a rate of 1200 pictures per second. It must be remembered that in this scheme of photography the axis of each picture shifts 12 degrees clockwise, making a complete revolution in thirty frames. Part of the change in position of eggs is

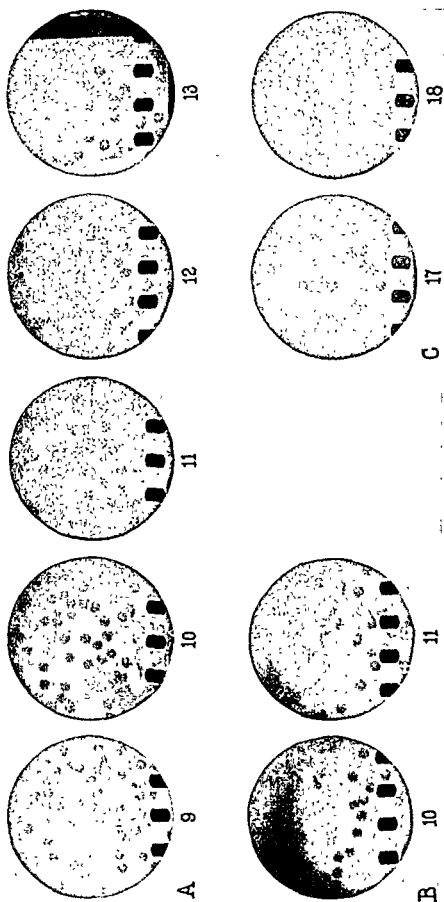


FIG. 3. Photographs of unfertilized eggs of the sea urchin, *Arbacia*, subjected to high frequency sound waves, each picture taken $1/1200$ second apart. Exposure about one millionth second. The numbers give the picture sequence. The sound waves were started at the sixth picture. A, B, and C are different series of pictures.

due to the shift of the axis. Several points of interest appear in these pictures.

First the disturbance which results in destruction is decidedly local, as illustrated by A where the second frame shows complete disintegration of a clump of eggs followed by vortex movements in the field. The localized effect of supersonics is frequently observed when fertilized eggs in the two or four cell stage are observed. Only one of two blastomeres may be disintegrated and the other remain unharmed.

Second, eggs in one frame may be unharmed and in the next completely disintegrated, (Fig. 3 A 9 and 10) or,

Third, eggs may be drawn out into spindle or tadpole shaped bodies in one frame and in the next be completely disintegrated (Fig. 3 B and C).

Fourth, no cavitated air bubbles, which would be apparent as small black dots, are visible. It is of course possible that air bubbles too small to be visible may be present.

Fifth, rapid movements of the fluid change the position of the eggs from one frame to the next. This is especially noticeable where the boundary of the drop of eggs is photographed (not shown in the figure). Great changes in the form of the sea water air interface appear from one picture to the next. The movements are unusually rapid.

Sixth, the disintegration does not start the moment the supersonics are turned on. Part of the time may be due to starting the vibration in the crystal apart from the building up of oscillations in the electrical circuit. The supersonics are turned on in the sixth frame whereas in three films the pictures show normal eggs in the tenth while in the eleventh frame the eggs are in process of disintegration; in one film normal in ninth and a cloud of debris in tenth while in one film no disintegration is visible until the sixteenth frame. In later frames local explosions of groups of eggs, for that is the way they are best described, may occur. On the average there appears to be a lag of four frames or 1/300 of a second after plate voltage is applied to the oscillator before disintegration of eggs by supersonics occur, the actual disintegration taking less than 1/1200 of a second.

The mechanism of break-up is not so easy to interpret from the pictures. Although no cavitated air bubbles are apparent, a great deal of evidence indicates that rapid movements in fluids through

which sound waves pass are always connected with cavitation. Frequently, cavitation occurs with explosive suddenness under conditions where sound density has just the right value, giving rise to sudden extremely rapid movements in the fluid. The drawing of eggs into spindle and tadpole shape suggests that these movements, the result of cavitation, are the chief factor in the destruction of cells, rather than the puncture of the surface which might be due to the rapid vibration of a cavitated gas bubble.

SUMMARY

A new type of camera system is described capable of taking 1200 pictures a second through a microscope objective.

Photographs showing the destruction of *Arbacia* eggs by high frequency sound waves indicate that the disintegration occurs in less than $1/1200$ second.

Eggs drawn out into spindle or tadpole shapes suggest that rapid movements of the fluid tearing the eggs may be responsible for the disintegration. Although no cavitated air bubbles show in the photographs, other experiments make it likely that the rapid fluid movement is the result of submicroscopic cavitation.

We take pleasure in thanking Mr. Charles Butt, Research Assistant in Physiology, Princeton University, for his aid in taking these pictures.

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STUDIES ON CRYSTALLINE UREASE

IV. THE "ANTITRYPTIC" PROPERTY OF CRYSTALLINE UREASE*

By HENRY TAUBER AND ISRAEL S. KLEINER

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(Accepted for publication, August 17, 1931)

In a study published by one of us (1) it was stated that crystalline urease is inactivated by trypsin. Recently Waldschmidt-Leitz and Steigerwaldt (2) repeated this experiment and report that, contrary to our finding, crystalline urease is not inactivated by trypsin and therefore is not a protein as declared by Sumner and his associates and by us. This radical difference in the result of so simple an experiment demanded an explanation. Accordingly we have repeated the work and in offering the reason for this discrepancy we are able to present a very interesting and unsuspected property of crystalline urease.

We first repeated the experiment in exactly the manner described by Waldschmidt-Leitz and Steigerwaldt. We can confirm their results absolutely. That is, urease, whether crude or purified, is not inactivated by trypsin in a phosphate buffer of pH 7 (0.5 M). However, there was one condition of our earlier experimental work which Waldschmidt-Leitz and Steigerwaldt had failed to observe and which seemed unimportant. This was the addition of a gum. Since aqueous solutions of crystalline urease are extremely unstable, Sumner and Hand (3) in 1928 suggested the addition of gum arabic. This protects crystalline urease from inactivation even for several days. For this reason in the earlier work no experiments were done without the gum. We therefore have repeated the experiments with the addition of gum arabic and have been able to confirm our former results, namely that trypsin inactivates crystalline urease. The presence of a gum seems to be of great importance.

* This work was partly aided by a grant of the Committee on Scientific Research of the American Medical Association.

tions 2 ml. of urease with or without gum, were added. Controls were run by adding boiled trypsin to the urease gum solutions without inactivation of the urease. A typical series of experiments with trypsin is incorporated in Table I.

TABLE I

Experiments with Trypsin

All experiments performed at 37°

Experiment no.		Per cent inactivation of urease		
		After 1 day	After 2 days	After 3 days
1	Fairchild's trypsin plus urease in gum arabic solution	58	86	95
2	Fairchild's trypsin boiled plus urease in gum arabic solution	No inactivation	No inactivation	No inactivation
3	Fairchild's trypsin plus urease without gum	" "	" "	" "
4	Difco trypsin plus urease in gum arabic solution	52	83	90
5	Difco trypsin boiled plus urease in gum arabic solution	No inactivation	No inactivation	No inactivation
6	Difco trypsin plus urease without gum	" "	" "	" "
7	Dr. Northrop's trypsin plus urease in gum arabic solution	Complete inactivation within 8 hrs.		
8	Dr. Northrop's trypsin boiled plus urease in gum arabic solution	No inactivation after 3 days		
9	Dr. Northrop's trypsin plus urease without gum	No inactivation after 3 days		

The results are very striking; all three specimens of trypsin are capable of digesting or inactivating urease if gum is present, but not in the absence of gum. The rapidity of the action of a highly purified trypsin (Dr. Northrop's) should be noted. When experiments were repeated with gum ghatti similar results were obtained.

It became evident, too, that trypsin, both active and boiled, also can act as a protective colloid for crystalline urease. Although a simple aqueous solution of crystalline urease becomes inactive in 3 days or so, such a solution will remain quite active ureolytically if trypsin is present. It was this protective colloidal action of trypsin which permitted Waldschmidt-Leitz and Steigerwaldt to observe an activity of urease in aqueous solution (without gum) after 3 days.

EXPERIMENTAL

The trypsin solutions were prepared as described in the earlier (1) paper. 1 gm. of trypsin was dissolved in 90 ml. of phosphate buffer of pH 7 (0.5 M) to which 10 ml. of glycerol were added. Two commercial preparations were employed, Fairchild's and Difco. In addition we had the opportunity of using a highly active solution of crystalline trypsin (4) which Dr. J. H. Northrop very kindly furnished us. The latter was in 60 per cent glycerol and had an activity of about 160 units per mg. protein. Northrop defines a trypsin unit as the amount necessary to effect a change of 1 per cent in the viscosity of a 2.5 per cent solution of isoelectric gelatin at 35° in 1 minute (5).

The crystalline urease was prepared according to the method of Sumner (6), from the same jack bean meal as was used in some work recently reported (7). The crystals were centrifuged off from their mother liquor, dissolved in distilled water, and centrifuged again to free the solution from insoluble matter. The water solution was diluted further with water, gum arabic or gum ghatti, respectively, so as to contain 1 unit of urease per ml. A few experiments were done with 5 units of urease per ml., with the same results. A unit according to Sumner is the amount of urease which will produce 1 mg. of ammonia nitrogen from urea (3 per cent urea in 0.5 M phosphate buffer of pH 7) in 5 minutes at 20°. For the determination of urease activity Sumner's (1926) method was used.

Preparation of Gum Solutions.—Two different gums were used, gum arabic and gum ghatti. For the preparation of the gum arabic solution, 2 gm. of gum arabic (Baker) were dissolved in 100 ml. boiling distilled water, which yielded a colorless solution free of ammonia. The gum ghatti solution was prepared by putting 20 gm. of gum ghatti (Eimer and Amend) in four layers of cheese cloth (15 in. sq.) and suspending it in a graduate containing 500 ml. distilled water. It was left overnight and enough gum dissolved to make an excellent protective colloid. It was mixed and after standing a short while decanted, so as to get rid of small undissolved particles. Gum ghatti is always free of ammonia whereas some lots of gum arabic are not. Both gums were neutralized to pH 7.

Digestion Experiments.—To 2 ml. of Northrop's trypsin an equal amount of phosphate buffer of pH 7 (0.5 M) was added and to 2 ml. of this and 2 ml. of each of the other two trypsin-glycerol buffer solu-

in the literature about so called "Antitrypsins" (Hedin, 1905-1908; Young, 1918; Hussey and Northrop, 1923). The action of these substances is explained by Euler (9) in the following way: "The enzyme combines with substances more or less protein-like, as it does with its substrates which it splits. These substances can not be split or only very slowly. On account of this a part of the enzyme is deprived of its substrate and the action of the enzyme is retarded."

With these points in mind we suggest the following as a tentative explanation of our results. We must assume that different parts of the trypsin and urease molecules enter into combination under varying conditions. Thus an inhibitive group of urease combines to form an irreversible inactive compound with trypsin. This does not attack urease, hence presumably its proteolytic group is in combination. The ureolytic activity of this compound, however, is unchanged. If to crystalline urease, gum is added before trypsin, there is no formation of such a compound and tryptic digestion takes place. Possibly in the latter case the gum combines with part of the urease molecule and thus produces a configuration which does not permit of a combination such as is necessary to form the inactive trypsin-urease compound.

SUMMARY

1. Crystalline urease is not inactivated by trypsin in the absence of a gum. In fact, the presence of trypsin alone in aqueous solutions of urease has an action similar to that of gum, that is, it acts as a "protective colloid" for urease.

2. Crystalline urease is inactivated by trypsin in the presence of a gum. This occurs with great rapidity if purified (crystalline) trypsin is used.

3. If trypsin is added to urease a considerable time before the addition of gum no inactivation of the urease takes place.

4. The suggested explanation is that an inhibitive group of the urease molecule ("antitrypsin") may combine with trypsin to form an irreversible inactive trypsin compound, which cannot attack the urease but has unchanged ureolytic power. This compound cannot be formed if gum is present because the gum has united with a portion of the urease molecule; the tryptic power is thus unimpaired and urease is then digested. This speaks for the protein nature of urease.

oleate to all gelling systems, particularly among the proteins, is valid. In other words, is the identity of conductivity in the sol and gel state due to an identical micellar structure of these protein systems, or is it due to the conductivity of the excess electrolytes being so much greater than the conductivity of the ionized protein salts that the difference in conductivity produced by the structural changes involved in the sol-gel transformation was not detectable by the experimental procedure employed? That there is little or no change in the conductivity and diffusibility of electrolytes in colloidal systems on changing from sol to gel has long been known and is not a vital point in connection with the micellar theory of Laing and McBain, since all theories of gel structure postulate that the structure produced is enormous in size compared with ionic dimensions and thus exerts almost no hindering effect on ionic movement.

To obtain a fuller insight into the question, the following study has been carried out on the conductivity of gelatin sols and gels. Gelatin was selected because it is an example of a protein capable of undergoing a reversible sol-gel transformation. In carrying out the study we had in mind the work of Krishnamurti (8) who, from a study of the light-scattering in sols and gels of agar, has concluded that the micellar structures in the two states are not identical; and also that of Craig and Schmidt (9) who found differences between the refractive indices of gelatin sols and gels.

Experimental Procedure

In measuring the conductivity, the Kohlrausch principle was employed, with the difference that a one-stage vacuum tube amplifier was introduced between the bridge and the telephone, which made it readily possible to make measurements accurate to 0.1 per cent. The source of the bridge current was a General Radio Oscillator, and the capacity of the conducting cell was balanced in parallel by the setting of an adjustable condenser. The conductivity cell used throughout the experiments was of the bottle type, constructed of Pyrex glass. A thermometer, readable to 0.1° , was fitted into the neck of the cell in such a way that it could be immersed in the gelatin without interfering with the continuity of the liquid between the electrodes. All measurements were made at 25°C . "Difco" granular gelatin was used in the experiments. It was purified and made ash-free according to the procedure described by Loeb (10). The purified gelatin on analysis was found to be totally ash-free and in aqueous solutions to have a pH value of 4.75 as measured by the hydrogen electrode. The analysis of the gelatin

SOL-GEL TRANSFORMATION

asing conductivity for the sol and an increasing conductivity for gel, which on being allowed sufficient time, starting from either

TABLE I

The Conductivity of Ash-Free Gelatin Sols and Gels

gelatin	Sol specific conductivity mhos $\times 10^5$	Gel specific conductivity mhos $\times 10^5$	Difference	Remarks
<i>per cent</i>			<i>per cent</i>	
0.90	4.27	4.27	0	Sol stable at 25°
0.67	6.70	6.49	3.1	Gel stable form
0.17	7.21	7.05	3.2	Gel stable form
0.62	8.70	8.28	4.8	Gel stable form
0.58	13.60	12.68	6.7	Gel stable form

TABLE II

*The Influence of Electrolytes on the Conductivity of Gelatin Sols and Gels**

electrolyte	Concentration N	Gelatin	Sol specific conductivity mhos $\times 10^5$	Gel specific conductivity mhos $\times 10^5$	Difference
		<i>per cent</i>			<i>per cent</i>
0	0	2.80	9.98	8.96	10.2
Cl	1.25×10^{-4}	2.80	11.32	10.14	10.0
Cl	1.24×10^{-3}	2.75	19.10	18.65	2.3
Cl	1.14×10^{-2}	2.55	99.70	99.70	0
0	0	2.70	9.44	9.73	7.6
OH	9.79×10^{-5}	2.70	10.00	9.90	4.1
OH	9.70×10^{-4}	2.65	15.55	15.00	3.8
OH	8.90×10^{-3}	2.45	53.25	53.25	0
0	0	2.90	9.81	8.82	10.1
Cl	8.85×10^{-5}	2.90	12.50	12.10	3.4
Cl	8.77×10^{-4}	2.90	22.15	21.75	1.9
Cl	8.05×10^{-3}	2.65	98.35	97.90	0.4

he lack of correspondence to a small degree between the gelatin concentration and the conductivity in certain of the experiments listed is explained by the drift in conductivity noted in the text which makes an exact reproducibility in these solutions impossible.

sol or gel state, finally reached a constant value representative of the equilibrium state of the gelatin at that temperature. In the

present experiments, this equilibrium state for all except the 0.9 per cent gelatin of Table I was a firm gel. In the experiments where difference in conductivity was found between the sol and gel condition this drift was absent and in these samples when the thermostat temperature was once attained, the conductivity became constant. It was the result found for the first experiment of Table I, with a concentration of 0.90 per cent gelatin for which a semiliquid state was the state at 25° and for the experiments of Table II where the conductivity difference between sol and gel was zero although firm gels were stable at the 25° temperature. The conductivity figures for the rest of the experiments in Tables I and II are the values measured just after the thermostat temperature was attained by the gels.

The data with the pure gelatin and the electrolyte-containing gels are not in accord with McBain's theory. Rather they are in harmony with the viewpoint that there is a distinct difference in micellar units of the sol and gel state when a definite firm gel is formed. The gel state shows the lower conductivity of the two forms, which would be expected if the micellar unit of the gel is composed of aggregates of the sol micelles, thus naturally resulting in a lower electrical mobility. That the difference in conductivity is due mainly to electrical changes accompanying the structural changes of the sol-gel transformation is indicated by the experiments of Table I. Since in these experiments, the gelatin was completely ash-free, the measured conductivity can only be a measure of the electrical charge of the gelatin in the solution and the accompanying hydrogen ions. From the pH value of 4.75 given by these solutions, the hydrogen ion concentration is less than 2×10^{-6} mols per liter, which, using the value of 350 for the hydrogen ion mobility, leads to the value of 0.7×10^{-6} for the specific conductivity. This value in comparison with the values found for the gelatin, points to the conductivity coming mainly from the charged gelatin and that the decrease in conductivity on gelation is due to aggregation of the gelatin units.

The data of Table II substantiate this point of view. When the electrolyte concentration is low there is a distinct difference between the conductivity of the sol and gel state. This difference decreases with increase in the electrolyte concentration and is no longer detected when the conductivity of the electrolyte-containing solutions increa

to about 100-fold the value of the difference in the conductivity between the sol and gel of the original isoelectric gelatin.

The conductivity difference between the gelatin sols and gels as shown in Table II becomes undetectable at an electrolyte concentration of approximately 0.01 molal, yet blood and protoplasm contain more than tenfold this amount. On this account, the experiments on the electrolyte-containing gelatin solutions throw grave doubts on the conclusion drawn by Gelfan for protoplasm and by Gelfan and Quigley for the blood coagulation process. In view of the large excess of free electrolytes in the systems studied by the above authors, the fact that they found no change in conductivity with changes in viscosity or on gelation, is, under the circumstances, no proof of either an identity of micellar structure in the different physical states of the systems they studied or of a micellar structure at all.

From the present studies, along with Craig and Schmidt's refractometric results, and the work of Krishnamurti on agar, it must be concluded that the McBain theory of an identical unit structure for the sol-gel state has no general applicability.

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ON THE RATE OF OXYGEN CONSUMPTION BY FERTILIZED AND UNFERTILIZED EGGS

I. *FUCUS VESICULOSUS*

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The rate of oxygen consumption, and the change in rate following fertilization, has been measured in a number of animal eggs. This change in rate has become a matter of some interest in connection with the nature of fertilization, especially since Warburg's (1) discovery in 1908 that there is a six- or sevenfold increase following fertilization in the sea urchin egg. Loeb and Wasteneys (8) extended the work to include other eggs, as have a number of workers since then. Loeb based his hypothesis of fertilization largely on a generalization of the respiratory phenomenon of the sea urchin egg, in conjunction with other work of his own on fertilization and artificial parthenogenesis, and especially on the rôle of cytolysis. A general discussion of the work in this field, and the relation to it of the present work, is included in another paper (2).†

The eggs of the alga *Fucus* resemble animal eggs in a number of respects, although they also have typical properties of the plant cell. In size and shape they resemble the eggs of the sea urchin *Arbacia*, although being both smaller on the average (diameter average = 65 microns), and more variable in size. They contain chlorophyll, in plastids, and secrete a jelly following fertilization which soon becomes a cellulose wall. Like the egg of *Arbacia*, the *Fucus* egg contains a single haploid nucleus, which in *Fucus* usually lies in the center of the cell. Fertilization is normally accomplished with the entrance of one sperm or antherozoid. The group of plants to which

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† The fourth paper of this series.

Fucus belongs is unusual in that the haploid generation of the life cycle is confined to the period between maturation of the gametes and fertilization, resembling in this respect the common condition in animals. Like most animal eggs the fertilized *Fucus* egg develops an amphiaser. Some species of *Fucus* are hermaphroditic, but *Fucus vesiculosus* is dioecious. The gametes are obtainable in abundance, especially during the winter and spring, and are comparatively hardy and viable under conditions of laboratory culture. Compared with certain other eggs however, the *Fucus* eggs are not especially well suited to manometric measurements of respiration. They are rather easily damaged by shaking, and the rate of respiration increases if they are damaged.

So far as I know the respiratory change associated with fertilization of a plant egg has not before been measured. Overton (3) has brought about artificial parthenogenesis in the eggs of *F. vesiculosus* with butyric acid. A more complete description of the *Fucus* egg, with reference to other experimental work on this form, is included in another paper (4).

Method of Handling and Fertilizing the Eggs

The plants were collected on the shore of Nahant peninsula near Boston, where they are abundant in the tide pools, growing attached to fixed igneous rock or to large boulders too heavy to shift with the tide. The plants were taken directly to the laboratory and kept thereafter at about 3°C. Usually on the day following the collecting, the gametes were extruded by the fruiting tips or receptacles which had been cut off and kept in moist chambers. It is important to keep the plants and the gametes cool.

Preliminary measurements of the rate of oxygen consumption showed that it would be advisable to use manometers designed to have a comparatively high ratio of fluid volume to gas volume, in order to facilitate measurement of small absolute rates of oxygen consumption. Warburg manometers were used. The vessels which were designed for the purpose are described in another paper (2).^{*} It was also found that, as might be expected, the eggs are photosynthetic. For the measurements in which this factor was to be ruled out, the work was done in a dark room. Measurements with known intensity of illumination were also made in the dark room.

It soon became evident that high percentages of fertilization were difficult to obtain. At the top side of each manometer vessel a small bulb in which dry sperm

* The fourth of this series.

or sperm suspensions could be placed communicated with the vessel in such fashion that the sperm could be mixed with the eggs by tipping the manometer. If the sperm had been in the bulb for some time, inseminations made in this way rarely resulted in more than 10 or 15 per cent fertilization. The sperm rapidly became ineffective when remaining in the bulb in a CO_2 -free atmosphere. Much higher percentages were obtained by opening the vessel, after measurements had been made of the rate with unfertilized eggs, and adding fresh sperm directly to the sea water containing the eggs. Opening the vessel at this stage necessitated a new start for measuring, and in consequence by this method no measurement was made covering the first 10 minutes following insemination. This period was measured by other methods: by using sperm in the side bulb and accepting low percentage fertilization, and by placing the sperm in the side bulb only 10 minutes before insemination but after a prolonged measurement of the prefertilization rate. Sperm which had been in the bulb only 10 minutes before insemination (by tipping) fertilized a larger percentage of the eggs than sperm which had been in the bulb throughout the hour or more during which the prefertilization rate of the eggs was measured.

Eggs were found to be subject to fertilization and to normal development when more than a week old provided they had been kept at about 3°C . At high temperatures cytolysis sets in and parasitic organisms develop extensively. Spermatozoa or antherozoids were found to remain effective for 2 or 3 days or longer if kept dry, that is, if kept in the concentrated condition in which they are exuded, without addition of sea water. As soon as the concentrated antherozoids are placed in sea water the thin walled capsules in which 64 antherozoids are packed, dissolve. The liberated antherozoids, which have so far been quiescent, soon begin to swim actively, and their ability to fertilize eggs drops off markedly after an hour or two of active swimming, although it does not cease altogether for a longer time. In these experiments most of the eggs used were between 6 and 12 hours emerged from the capsules; none were older than 3 days. The sperm were always fresh and active. In all cases control inseminations were used to test the gametes.

Most of the experiments were carried on in a thermostat at 18°C . At this temperature the time-lapse between fertilization and 50 per cent cleavage is about 15 hours. That is, about 50 per cent of the fertilized eggs have completed the first cleavage after approximately 15 hours. The time-span of first cleavages in a population is large in terms of hours, but not so large, compared with common marine animal eggs, when counted as a percentage of the time-lapse from fertilization. The first cleavages took place in a population approximately during the time interval 13 to 18 hours after fertilization. A few sickly individuals took longer than 18 hours.

Method of Measurement

In the typical experiment four manometers were used. One served as a barometric control. Two contained approximately similar amounts of unfertilized

eggs. Later one of these was inseminated, after the prefertilization rate had been measured, and the other was used as an unfertilized control to test for continued constancy, or near constancy. The fourth vessel contained only sea water to which sperm were added, and in like amount, when one of the egg-containing vessels was inseminated. This served as a control for the respiration of the sperm which was always found to be negligible in the concentrations used in insemination.

In measurements of the respiration rate of high concentrations of sperm alone, the barometric was the only control. After an experiment the approximate volume of the eggs, and of the sperm in certain cases, was measured by centrifuging the contents of the vessels in calibrated vaccine tubes until the gametes failed to compress further. Values could be read to 1 c.mm. Since there is no doubt some free space among the packed eggs, the observed volumes are probably a little too great. This error is probably not large, and is at least fairly constant for different experiments. The vessels contained 2000, 3000, or 4000 mm.³ sea water, in which were from 5 to 45 mm.³ eggs. Most experiments involved between 8 mm.³ and 15 mm.³ eggs. Higher concentrations of eggs were found to suppress the rate of oxygen consumption, unless the rate of shaking was increased to an extent which damaged the eggs. The vessels were operated in an automatic water bath thermostat at 18°C. ± 0.02 . Each vessel contained 0.5 cc. 5 per cent KOH in a bulb or well to absorb CO₂. Following Warburg (5),

$$X = h \left[\frac{V_g \frac{273}{T} + V_F \alpha}{P_s} \right]$$

Where $X = \text{mm.}^3 \text{ O}_2$ consumed, $h = \text{change in pressure (observed) in mm. of manometer fluid (Brodies' fluid) at constant volume}$, $V_g = \text{volume in mm.}^3 \text{ of the gas space from the surface of the sea water to the meniscus}$, $V_F = \text{fluid volume in mm.}^3$, $\alpha = \text{solubility coefficient of oxygen in sea water (Bunsen's coefficient)}$, $P_s = \text{standard pressure in mm. manometer fluid (10,000 mm. Brodie fluid} = 760 \text{ mm. Hg)}$.

The Rate of Oxygen Consumption of Unfertilized Eggs in the Dark

The *Fucus* eggs, like sea urchin eggs, are almost naked before fertilization. The enclosing membrane is exceedingly delicate. This is perhaps one reason why they, like sea urchin eggs, are sensitive to shaking. The observed rate of respiration increases steadily and shaking is rapid. This is presumably due to damage to the shaking is so rapid as to cause a rising respiratory rate, integration are presently brought about, although if ved before being damaged to this extent they will by after insemination. Too slow a rate of shak-

ing on the other hand suppresses the observed respiration by failing to maintain gas equilibrium between the sea water and the air space above it. An optimum rate was found which gave repeatedly in different experiments approximately constant readings on any given set of unfertilized eggs for several hours, with neither a gradual rise nor a decline. It is possible that this amounted to finding a position of compensating errors rather than true elimination of errors, but it probably gives a value close to the true one. The eggs were in good condition, and developed normally when inseminated after several hours of shaking at this rate. The optimum rate with the vessels

TABLE I

Unfertilized Eggs. The Rate of Oxygen Consumption in $\text{Mm.}^3 \text{O}_2$ Per Hour Per 10 Mm.^3 Eggs

Experiment	Rate	Experiment	Rate	Experiment	Rate	Experiment	Rate	Experiment	Rate
1	4.4	9	4.7	17	6.4	25	4.6	33	6.8
2	8.1	10	3.8	18	4.4	26	6.0	34	6.7
3	3.8	11	4.6	19	6.2	27	5.9	35	5.1
4	7.4	12	7.3	20	5.9	28	5.7	36	6.8
5	6.1	13	8.3	21	3.8	29	5.4	37	3.6
6	5.0	14	5.1	22	4.7	30	5.9	38	4.0
7	5.0	15	4.6	23	4.9	31	5.8	39	4.8
8	4.8	16	4.4	24	4.1	32	6.4	40	5.1
Average rate.....									5.4

used was found to be 36 or 38 "round trip" shakes per minute, with an amplitude of 7 cm. Many measurements were made because the results are a good deal more variable than those of similar measurements on certain other eggs such as those of *Chaetopterus*. The first twenty-seven have been completely discarded as they involved faults of technique that were later eliminated or reduced. The results of the next forty measurements have been calculated in terms of the absolute rate: $\text{mm.}^3 \text{O}_2$ per hour per ten mm.^3 eggs. Each of these consists of the average of a number of consecutive measurements of a duration of from 10 minutes to half an hour, or in some cases an hour, depending on the circumstances, especially upon the con-

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The *Fucus* eggs, like sea urchin eggs, are almost naked before fertilization. The enclosing membrane is exceedingly delicate. This is perhaps one reason why they, like sea urchin eggs, are sensitive to shaking. The observed rate of respiration increases steadily and greatly if shaking is rapid. This is presumably due to damage to the eggs. If the shaking is so rapid as to cause a rising respiratory rate, cytolysis and disintegration are presently brought about, although if the eggs are removed before being damaged to this extent they will often develop normally after insemination. Too slow a rate of shak-

zooids began to drop off. After 2 hours it may have become only one quarter of the initial rate. The absolute rate of O_2 -consumption for swimming antherozoids, as measured in $mm.^3 O_2$ per hour per 10 $mm.^3$ antherozoids, is very high compared with the rate for the eggs. The rates measured in two good experiments, with 13.5 $mm.^3$ antherozoids in 4000 $mm.^3$ sea water, are given in Table III. In these experiments the rate has begun to drop off 75 minutes after the start of the measurements. The average rate in both experiments for the first five measurements is 25.5 $mm.^3 O_2$ per hour per 10 $mm.^3$ antherozoids. This is nearly five times the rate of the unfertilized eggs.

TABLE III

Oxygen Consumption of 13.5 Mm.³ Antherozoids in 4000 Mm.³ Sea Water in Mm.³ O₂ Per Hour Per 10 Mm.³ Antherozoids

Time, beginning 20 min. after antherozoids were first placed in sea water	Rate, Vessel 1	Rate, Vessel 2
<i>min.</i>		
0-15	25.8	25.2
15-30	26.2	25.3
30-45	25.7	25.3
45-60	25.8	24.8
60-75	25.3	25.3
75-90	21.7	21.6
90-105	19.3	19.2
Average rate in both vessels for first 75 min.		25.5

Eight experiments were designed to find out if the antherozoids, with a great preponderance of carotinoids in the pigment content, are photosynthetic. The results were inconclusive, but in one of the experiments especially it appears that the overall rate of oxygen consumption is less with an illumination of 100,000 foot candles than in the dark, to the extent of something like 10 per cent. This might, of course, be due to an effect of light on the rate of swimming. Cultures of antherozoids were observed with bright and dim lights, and no difference could be seen in the rate of swimming, but a change of 10 per cent would not have been detected. The results suggest a certain amount of photosynthesis but are hardly conclusive.

given set of eggs was used only once. Every part of the period was covered by a number of measurements.

It is more difficult to determine accurately the percentage fertilization in *Fucus* eggs than in most animal eggs. In the first place, while the *Fucus* egg secretes a membrane following fertilization it is scarcely visible with simple microscopic examination. The best criterion is to count samples after division of the eggs. This does not take place for many hours, and unless nearly all of the eggs divide, it is always possible that some eggs which were activated have fallen by the way before cleavage time. Shrinking the eggs a few hours after insemination by the addition of sugar to the sea water reveals the membranes of the fertilized eggs much more clearly, and was found to be useful in conjunction with subsequent cleavage counts of samples to which no sugar was added. There is further a tendency for selection when a sample of eggs is taken from an inseminated vessel, since the fertilized eggs tend to attach to the walls of the vessel as well as to each other in floating clumps. The eggs which failed to fertilize float freely, and an undue proportion of them may be taken in the sample. This can partly be guarded against by first scraping most of the attached eggs from the walls of the vessel, although a number will be destroyed in the process. All of these difficulties apply only to cases in which high percentage fertilization failed to take place. Most of the results are in this category, but there are enough cases with 95 per cent fertilization to serve as a standard. In view of these errors which apply when lesser percentages fertilization resulted, most of them tending to underrate the true percentage, it is perhaps not surprising that in some experiments with comparatively low observed percentage fertilization, the rate is higher in proportion than in the cases with 95 per cent observed fertilization. It is as if the percentage fertilization or activation were somewhat higher than the observed percentage, and this is very probably the case. Table IV represents the observed percentages fertilization and the observed average rate of oxygen consumption for the first hour following insemination, expressed as a percentage of the prefertilization rate of the same sample of eggs. The experiments are listed in the order of percentage fertilization. These averages for the first hour are made up of consecutive measurements over this period, the individual readings

being at intervals of from 5 to 15 minutes. Most of the experiments begin 10 or 15 minutes after insemination, and the first measured hour is then included in the average. In four cases with lower percentage fertilization, the first reading covers the period 0-10 minutes after insemination. The rate is constant throughout the period 0-60 minutes after fertilization within the limits of measurement. The increased rate takes effect so soon after insemination that there is no

TABLE IV

The Average Rate of Oxygen Consumption for the First Hour after Fertilization Expressed as a Percentage of the Rate before Fertilization

Experiment	Fertilization observed	Rate of O ₂ consumption as per cent unfertilized rate
	<i>per cent</i>	
1	95+	194
2	95+	193
3	95+	188
4	95	175
5	95	182
Average.....		186
6	80	169
7	70	168
8	60+	195
9	45	152
10	40+	157
11	40	148
12	36+	147
13	15	145

measurable difference in the amount of oxygen consumed in the period 1-11 minutes and the period 11-21 minutes after insemination.

In Table IV it is seen that the average rate of fertilized eggs for the first hour of the five cases in which 95 per cent or more of the eggs subsequently cleaved in normal fashion, is 186 per cent of the pre-fertilization rate. The eight cases with lesser percentage fertilization substantiate this result. It appears then that the rate of fertilized eggs is of the order of 190 per cent of the rate of unfertilized eggs.

Repeated overlapping measurements cover the period between 1

and 24 hours after fertilization. The rate is constant throughout this period up until about the early middle of the first cleavage period, *i.e.*, until about 13 or 14 hours. From this time on a very gradual slight increase in the rate takes place. This is shown in Fig. 1. After the appearance of the first cleavage there is no cessation of cleavages in a population. The second cleavage of the earlier dividers takes place before the first cleavages of the late dividers, so that there is an out-of-phase overlapping of cell divisions. For many hours after fertilization the eggs are to all external appearances in a resting condition. The mitotic phenomena proceed very slowly after the pro-

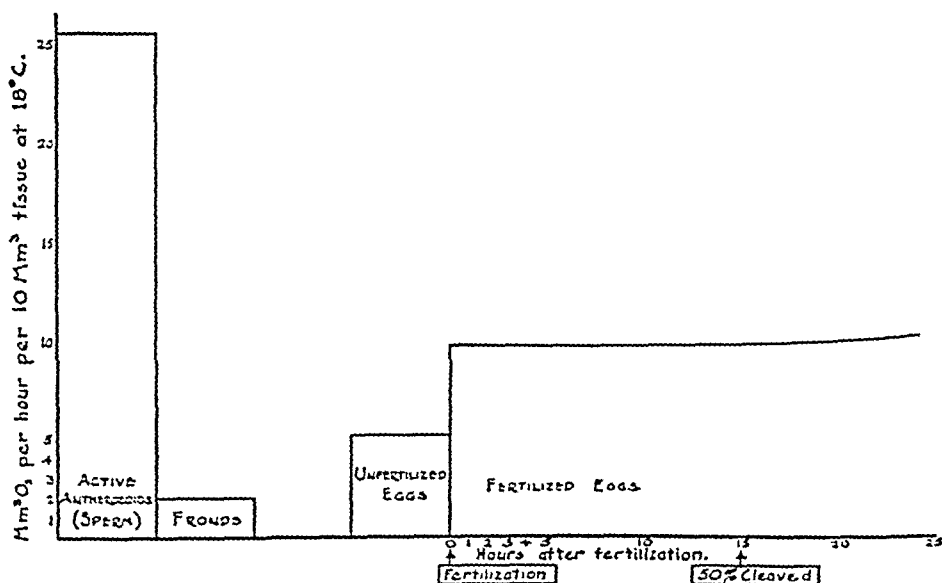


FIG. 1. *Fucus vesiculosus* in the dark.

nuclei have come together (Farmer and Williams (7)). The fact that some spores cleave 4 hours sooner than others indicates rather strongly that certain precleavage processes are by no means in a state of temporary suspension, as 4 hours would represent an astonishing difference in rate in different individuals unless it is the accumulated difference in progress of the full 15 hours of activity. The gradual increase starting at about the time of cleavage and continuing thereafter is exceedingly slight and probably represents developments which may be classified as growth, which is very slow in the *Fucus* spore.

DISCUSSION

The rate of oxygen consumption by the *Fucus* egg, when it is unfertilized and in a resting condition, is high compared with that of eggs of marine invertebrates. Thus it is of the order of ten times the rate in the unfertilized egg of the sea urchin *Arbacia*, per unit volume, and more than one and a half times the rate in the egg of the clam *Cumingia*. The eggs of *Arbacia* and *Cumingia* are of about the same size as the *Fucus* egg, and represent respectively the lowest and highest respiratory rates of several unfertilized eggs of marine invertebrates including *Arbacia*, *Nereis*, *Chaetopterus* and *Cumingia* (2).

In the *Fucus* egg the time-lapse from fertilization to the first cell division is so long, and the mitotic preparations for cleavage are so slow, that a measurement of the change in rate of oxygen consumption immediately following fertilization is perhaps especially free from effects of the precleavage processes which proceed at so much more rapid a rate following fertilization in most animal eggs. The change in rate may pertain more strictly or more exclusively to the activation of the eggs as such, or to the physiological changes which are brought about by this activation. It should not be implied however that cleavage and the changes in the cell which precede it are distinctly divorced from the chemical changes involved in activation.

The *Fucus* egg increases its rate of consumption of oxygen abruptly at fertilization. The exact length of the period of transition in rate is not determined, but it is in all probability very short. This abrupt change in the rate of respiration, comparable to similar changes in a number of animal eggs, suggests an essential similarity, in certain respects at least, in the nature of fertilization. This is in a general way in keeping with Overton's (3) discovery that parthenogenesis of the *Fucus* eggs may be induced by treatments which activate sea urchin eggs (notably with butyric acid).

SUMMARY

1. The unfertilized eggs of *Fucus vesiculosus*, in the dark, consume about 5.2 mm.³ O₂ per hour per 10 mm.³ eggs.
2. With an illumination of 100,000 foot candles in photosynthesis they liberate more than twice as much oxygen as they consume.
3. The actively swimming antherozoids or sperm of *Fucus* consume

oxygen at a very high rate: 25.5 mm.³ O₂ per hour per 10 mm.³ antherozoids.

4. Immediately following fertilization, in the dark, the *Fucus* eggs increase the rate of oxygen consumption to about 190 per cent of the prefertilization rate.

5. This rate for fertilized eggs, about 190 per cent, is maintained uniformly for 13 or 14 hours, after which there is a barely perceptible rise until 24 hours (when measurements ceased). At 18°C. 50 per cent of the spores in a population have completed the first cell division about 15 hours after fertilization.

I am much indebted to Professor W. J. Crozier, in whose Laboratory this work was carried out, for valuable advice and criticism. I am also indebted to Dr. Robert Emerson for advice regarding certain phases of the Warburg technique.

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ON THE RATE OF OXYGEN CONSUMPTION BY FERTILIZED AND UNFERTILIZED EGGS

II. CUMINGIA TELLINOIDES

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(Accepted for publication, September 9, 1931)

Measurements have been made of the rates of oxygen consumption by the eggs of several marine forms, and especially of the change in rate following fertilization. It is convenient to present some of these results separately, considering them comparatively in one of the papers and discussing the relation to other work in the field, and some of the theoretical implications. This will be done in the fourth paper (1) of this series. The present paper will be chiefly concerned with measurements on the eggs of the clam *Cumingia*.

In the summer of 1930 measurements of the rate of oxygen consumption of *Chaetopterus* eggs showed that immediately following fertilization there is a decided *decrease* to slightly more than half of the prefertilization rate. This surprising result was entirely unexpected, and many careful repetitions of the measurements were made, which invariably confirmed the first finding. When the eggs of *Chaetopterus* are taken from the parapodia of the female and are placed in sea water, dissolution of the germinal vesicle takes place and maturation proceeds until the nucleus comes to rest in the metaphase of the first polar spindle. This takes less than 15 minutes at 20°C. In this stage the egg rests until it is fertilized. When the unfertilized eggs are placed in manometers after having attained the metaphase resting condition, the rate of oxygen consumption is uniform for at least 8 hours. Fertilization at any time during this period (which was the longest experimental period) causes a sharp drop to about half.

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oxygen at a very high rate: 25.5 mm.³ O₂ per hour per 10 mm.³ antherozoids.

4. Immediately following fertilization, in the dark, the *Fucus* eggs increase the rate of oxygen consumption to about 190 per cent of the prefertilization rate.

5. This rate for fertilized eggs, about 190 per cent, is maintained uniformly for 13 or 14 hours, after which there is a barely perceptible rise until 24 hours (when measurements ceased). At 18°C. 50 per cent of the spores in a population have completed the first cell division about 15 hours after fertilization.

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vessel served as a control for determining the respiration of the sperm. It might be objected that the sperm in the vessel containing eggs, because of the eggs and their secretions, are probably not in the same state of activity as those in the control vessel. However, in all cases, as well as in measurements on *Fucus*, *Chaetopterus*, and *Nereis*, the respiration of the amount of sperm used in insemination was found to be entirely negligible. Each vessel also contained 0.2 cc. 5 per cent KOH solution in a bulb or well to absorb CO_2 .

One of the two vessels containing eggs was used as an unfertilized control to be sure that the treatment was such as to give approximately constant respiration. The other egg-containing vessel, run simultaneously, was used at first for measurement of the unfertilized rate. After this had been obtained reproducibly for several readings the eggs in this vessel were inseminated, and measurements were continued. After an experiment the volume of the eggs was determined by centrifuging the contents of each vessel in an accurately calibrated vaccine tube, in which volume could be read to about 1 c.mm. It was found that if the eggs are centrifuged at a certain speed for 10 minutes, additional centrifuging causes no further measurable decrease in the volume which the packed eggs occupy. Readings of the volumes of eggs were made after centrifuging for 15 minutes. Since there is no doubt some free space among the packed eggs the observed volumes are probably a little too great, but this error is probably not large and it is at least about the same for different experiments.

The Conditions of Measurement

The manometers were mounted on a shaker of adjustable rate and amplitude. A certain optimum of these adjustments must be found by preliminary experimentation. Too rapid a rate of shaking damages the eggs, while too slow a rate does not maintain equilibrium of gas tension between the sea water in the vessel and the air above it. In the cases of the eggs of *Fucus* and *Arbacia* it was found that this optimum rate of shaking is quite crucial. Small deviations cause on the one hand a gradual rise, with cytolysis, or if the shaking is a little too slow, a gradual decline in the rate of respirations of unfertilized egg. It is possible, however, to find a rate and amplitude of shaking which gives constant readings for a long time. The eggs of both *Cumingia* and *Chaetopterus* are much more favorable to work with in this respect. A greater range of shaking speed continues to give constant readings. This may be largely due to the tough membrane which envelops these eggs, which probably permits them to withstand a greater rate of shaking without damage. The optimum speed of shaking is of course also a function of the size and shape of the vessels. For these experiments a rate of forty-eight round trip shakes per minute and an amplitude of the vessel of 7 cm. gave constant readings on unfertilized eggs for several hours.

The manometers were submerged in an aquarium full of stirred water which had been arranged as a thermostat. The temperature was kept at $21^{\circ}\text{C}.$, ± 0.05 .

The prefertilization rate is not regained until several hours after fertilization.

The eggs of the clam *Cumingia* undergo nuclear changes before fertilization which almost exactly parallel the changes in *Chaetopterus*. When the eggs are shed through the siphon into sea water, they are immature. In the water the germinal vesicle quickly breaks down, liberating nuclear sap into the cytoplasm, and the chromosomes line up in the metaphase of the first polar spindle. In this condition the eggs rest until they are fertilized. The eggs are small, averaging about 65 microns in diameter. They are enclosed in a tough membrane, even before fertilization (as demonstrated with the micro-needle), and withstand manometer shaking very well.

THE EXPERIMENTS

The experiments with the *Cumingia* eggs were performed at the Marine Biological Laboratory, Woods Hole, in August, 1930. For an experiment about 30 clams were washed and placed each in a separate Syracuse dish containing sea water. When these animals have been kept out of water for a time after collecting, some of them will shed gametes after being returned to sea water. The males usually begin to shed in about 25 minutes or half an hour, if in good season, and the females somewhat later. Unlike the measurements on *Chaetopterus*, in which one female shed enough eggs for a complete experiment, the measurements on *Cumingia* were made on the mixed eggs of several females. In order that all the eggs would be in the same stage, and of approximately the same age, they were so selected that at the start of an experiment all eggs had been shed at least half an hour and none more than 50 minutes. After the eggs have been shed into sea water the prefertilization nuclear changes take about 10 or 12 minutes. The eggs were thoroughly washed at least twice to remove mucous and foreign matter, and were then concentrated after settling by removing sea water from above them with a siphon. Care was taken to avoid compressing or damaging any of the eggs in this process.

Warburg manometers were used. Four small rectangular vessels had been especially designed to facilitate measurements with small amounts of eggs. These vessels are described in another paper (1). In some experiments 4 cc., in other experiments 2 cc., of sea water and eggs were placed in each of two of the vessels. Approximately equal egg concentrations were obtained in the two vessels by filling the measuring pipette from the same well stirred dish of eggs and sea water. A third vessel contained a like volume of sea water and served as the barometric control. The fourth vessel contained a like volume of sea water to which exactly the same amount of sperm was added as was used to fertilize the eggs. This

vessel served as a control for determining the respiration of the sperm. It might be objected that the sperm in the vessel containing eggs, because of the eggs and their secretions, are probably not in the same state of activity as those in the control vessel. However, in all cases, as well as in measurements on *Fucus*, *Chaetopterus*, and *Nereis*, the respiration of the amount of sperm used in insemination was found to be entirely negligible. Each vessel also contained 0.2 cc. 5 per cent KOH solution in a bulb or well to absorb CO_2 .

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The manometers were submerged in an aquarium full of stirred water which had been arranged as a thermostat. The temperature was kept at 21°C , ± 0.05 .

Fertilizing the Eggs

The vessels all had small bulbs at the top of one side in which dry sperm or sperm suspension could be placed, later to be mixed with the eggs by tipping the manometer. It was found, however, in the case of *Cumingia* (as well as with *Fucus*, *Chaetopterus*, *Nereis*, and *Arbacia*) that after sperm have been in this side chamber even for so brief a period as 25 minutes, a high percentage of fertilization is not usually attained. I had earlier thought this to be due either to the effect of concentrated egg water on the sperm, or to damage to the eggs in shaking. But neither of these are the cause, since when vessels are opened and fresh sperm are added 95 per cent to 100 per cent fertilization often results, even if the unfertilized eggs have been shaken for several hours. It is more difficult to obtain good fertilization of *Fucus* and *Arbacia* eggs with sperm which have been kept in the manometers than of the other forms mentioned, especially *Chaetopterus*. The cause of the deterioration of the sperm in the bulb was not investigated. The difficulty was avoided by measuring the rate before fertilization, and then opening the vessels and adding fresh sperm to the egg vessel and to the sperm control vessel. Since opening the vessels changed the temperature, and the meniscus settling, a new start had to be made. No reading could be made covering the first 10 minutes after fertilization. The first measurement after fertilization covered the second 10 minute period.

It is also possible to make measurements covering the period immediately following fertilization in the following way: measurements are made on the unfertilized eggs until a reliable and reproducing value is gotten. This will usually consist of a 15 minute period at the start for the vessels to come to the temperature of the thermostat, and then perhaps four consecutive readings at intervals of 10 or 15 minutes (depending on the magnitude of the consumption, which depends among other things on the volume of eggs in the vessels). If the vessel to be inseminated is then taken from the tank and fresh sperm are quickly put in the bulb, the vessel may be returned to the thermostat with little handling and after having been out of the tank less than a minute. 8 or 10 minutes in the thermostat will return the vessel to thermostat temperature. If now the vessel is tipped to mix the sperm with the eggs and a reading is made, the next reading will

measure the period beginning at fertilization. The object of this procedure is to keep the sperm in the bulb only 10 or 15 minutes while allowing more than an hour, or an indefinite period, for measurements on the unfertilized eggs. By this method high percentages of fertilization were obtained with *Chactopterus* eggs. Only one such experiment was performed on *Cumingia* eggs and in this case resulted in only 35 per cent fertilization. Repetition would no doubt better this. The present experiments were not designed to test precisely how soon after insemination the change in respiration takes place.

RESULTS

The calculations of oxygen consumption were made following Warburg (2) for the simpler case in which CO_2 is eliminated by absorption in 5 per cent KOH:

$$X = h \left[\frac{V_g \frac{273}{T} + V_F \alpha_{o_2}}{P_o} \right]$$

where X = oxygen consumed in c.mm., h = change in pressure at constant volume in mm. of manometer fluid (Brodie's fluid), V_g = volume of gas space in c.mm. between the surface of the sea water and the meniscus, V_F = volume of the fluid (sea water and eggs) in c.mm., α_{o_2} = the solubility coefficient (Bunsen's coefficient) of oxygen in the fluid (sea water), P_o = standard pressure in mm. of manometer fluid (10,000 mm. Brodie = 760 mm. Hg.) For any given vessel at constant temperature and with a constant V_F the term within the bracket is a constant. The results have been calculated in terms of the rate: c.mm. of oxygen per hour per 10 c.mm. of eggs.

Measurements of the rate of consumption of oxygen by unfertilized eggs, each measurement covering a span of from 10 to 100 minutes, and being the average of consistent 10 minute consecutive readings over that period, are tabulated in Table I.

The average absolute rate of consumption by unfertilized eggs is seen to be 3.1 c.mm. of oxygen per hour per 10 c.mm. of eggs.

In five experiments, four of which had good controls, the rate after fertilization has been calculated as a percentage of the rate of the same eggs before fertilization. The simultaneous unfertilized con-

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The average absolute rate of consumption by unfertilized eggs is seen to be 3.1 c.mm. of oxygen per hour per 10 c.mm. of eggs.

In five experiments, four of which had good controls, the rate after fertilization has been calculated as a percentage of the rate of the same eggs before fertilization. The simultaneous unfertilized con-

trol of one experiment (Experiment 5, Table II) gave a consistent and standard result in the early part of the experiment but then had to be discarded because of a stop-cock leak. There is every reason to believe that the experimental vessel gave a good result. In those experiments in which the percentage of fertilization is considerably below 100 per cent it might be possible to extrapolate to 100 per cent on the basis of the observed percentage fertilization. This is dangerous, however, as it is at least possible that various unknown degrees of partial activation may have taken place which give no visible sign when the percentage fertilization is counted. In two cases of low percentage fertilization when extrapolation was attempted (Table

TABLE I

Experiment	Vol eggs in vessel*	Total duration of test	Mm. ³ O ₂ per hour per 10 mm. ³ eggs
		<i>min.</i>	
1	20 mm. ³	75	3.3
2	42 "	60	3.2
3	42 "	60	2.8
4	44 "	40	3.1
5	37 "	60	2.6
6	36 "	50	3.3
7	34 "	100	3.6
Average.....			3.1

* In 4 cc. sea water except in Experiment 5 in which there were 2 cc. sea water.

II, Experiment 3 and Experiment 4) this was evidently the case, the value obtained being explicable only on the assumption that a larger percentage fertilization or activation had actually taken place than was observed. If we do not make this assumption, it follows that fertilized eggs consume less than no oxygen, which is absurd. In another case, with somewhat better fertilization (Table II, Experiment I, 50 per cent fertilization) the value obtained by extrapolation to 100 per cent fertilization agrees closely with the direct result of two experiments of high percentage fertilization.

In five experiments the observed percentages of fertilization were 50 per cent, 95 ÷ per cent, 35 per cent, 15 per cent, 92 per cent. The

percentages were determined by sample counts involving in each case at least 200 eggs. It is perhaps best to consider the two cases of higher percentage fertilization separately, as they give a comparatively reliable result with slight correction by extrapolation, and then see to what extent the results are supported by the cases of low percentage fertilization. Fig. 1 shows with heavy line blocks the detailed results of the two best experiments averaged. They represent 92 per cent and 95 + per cent fertilization respectively. The dotted line blocks represent the simultaneous unfertilized control of one of

SOLID LINE = Average of 2 exp. with 96% and 92% fertilization.
DOTTED LINE = Simultaneous unfertilized control of one of these.
 [96% fert. exp.]

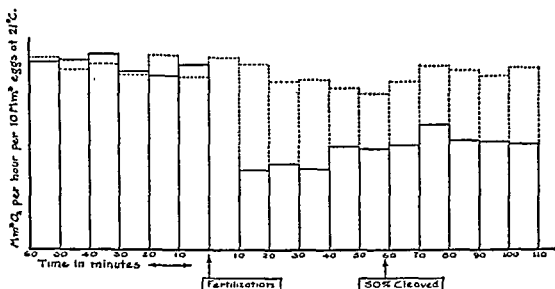


FIG. 1

these experiments (the other unfertilized control having been lost). The two results which are averaged are separately very similar.

Table II shows the results of the five experiments in tabular form.

These percentages (fertilized rate with respect to unfertilized rate, Table II) are calculated from the average rate of the first measured 50 minutes after fertilization, *i.e.*, of the period 10 to 60 minutes after fertilization, taken as a percentage of the rate for the same eggs before fertilization. It is seen that all data (without exception) point to a decrease after fertilization. The three cases in which the percentage fertilization is low are less reliable for giving an absolute

ON THE RATE OF OXYGEN CONSUMPTION BY FERTILIZED AND UNFERTILIZED EGGS

III. NEREIS LIMBATA

By D. M. WHITAKER*

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the Marine Biological Laboratory, Woods Hole)

(Accepted for publication, September 9, 1931)

INTRODUCTION

This paper is one of several giving the results of measurements of respiration before and after fertilization in a number of marine eggs including *Fucus*, *Chaetopterus*, *Cumingia*, *Arbacia* and *Nereis*.

A general discussion is reserved for another paper (1), the fourth of this series. The present report will largely be confined to the measurements on *Nereis* eggs.

The eggs of *Nereis* are unusual in several respects. They are fairly large, averaging nearly 140 microns in diameter. A few minutes after fertilization an immense amount of jelly is secreted by the egg; so much that one volume of eggs in sixteen volumes of sea water makes a firm jelly. Before fertilization the germinal vesicle is intact. About 18 minutes after fertilization (21°C.) the vesicles of most of the eggs of a sample have broken down. 45 minutes after fertilization about half of the eggs have the first polar body fully formed, and after 60 minutes, the second polar body. 50 per cent of the eggs have undergone the first cleavage at about 95 minutes. Lillie (2) has shown that the sperm does not actually enter the egg until about 40 or 50 minutes after insemination although it has activated the egg and instigated these changes by piercing the egg with its tip. When an attempt is made to cut the unfertilized *Nereis* egg with the microneedle it is found to be enclosed in an exceedingly tough membrane. This is probably one reason why *Nereis* eggs withstand shaking in a manometer exceptionally well. 99 per cent fertilization was obtained when sperm were added to eggs which had been shaking in a manometer for 4 hours.

Nereis were collected in the Eel Pond at Woods Hole in the months of August and September, 1930. At the beginning of an experiment the gametes were ob-

* National Research Council Fellow in the Biological Sciences.

value for the drop, but all show a decided decrease. The calculations for each experiment are based on the average of at least four consecutive measurements each before and after fertilization. The rate for the first hour after fertilization is about 45 per cent of the rate before fertilization.

Immediately after fertilization the rate is probably even less (see Fig. 1). The one experiment in which a measurement was obtained immediately after fertilization was the one with only 35 per cent fertilization. It indicated that the average rate for the first 10 minutes after fertilization is slightly lower than for the second 10 minute period.

TABLE II

Experiment	Percentage fertilization observed	Fertilized respiratory rate, as percentage of the prefertilization rate	Extrapolated rate, per cent fertilized
			per cent
1	50	71	41
2	95+	44	42
3	35	70	—
4	15	74	—
5	92	51	47

SUMMARY AND CONCLUSION

The rate of oxygen consumption by the eggs of the clam *Cumingia* decreases following fertilization, resembling in this respect a similar phenomenon of the eggs of the annelid *Chaetopterus*. The unfertilized *Cumingia* eggs consume approximately 3.1 mm.³ O₂ per hour per 10 mm.³ eggs. The average consumption of fertilized eggs in the period 10–60 minutes after fertilization is 1.4 mm.³ O₂ per hour per 10 mm.³ eggs or about 45 per cent of the prefertilization rate.

I am greatly indebted to Professor W. H. R. R. for the direction this work was done, for many helpful suggestions.

BIOGRAPHICAL

1. Whitaker, D. M., 1932, On the rate of oxygen consumption by unfertilized eggs. IV, (To be published).
2. Warburg, O., 1924, Verbesserte Methodik der Messung der kolyse. *Biochem. Z.*, 152, 51.

measured with reasonably consistent readings, the vessel to be inseminated was removed from the thermostat, opened with slight handling, sperm were added, and a like volume of sea water removed, and the vessel was quickly returned to the thermostat bath after being out less than a minute. The temperature of the water bath thermostat was $21^{\circ}\text{C} \pm 0.05$. Blank runs had shown that after this procedure ten minutes were adequate for the vessel to return to the temperature of the bath (which was very close to room temperature). The next reading was made 10 minutes after fertilization, and the second reading then gave a measurement covering the period 10-20 minutes after fertilization. In this way high percentages of fertilization were obtained while sacrificing measurement during the first 10 minutes following fertilization.

After extended measurements have given a value for the unfertilized rate, if instead of inseminating the eggs directly, sperm are now placed in the bulb, the 10 minute period which is lost for temperature equilibrium may precede fertilization. Following temperature equilibrium a reading is then taken and the manometer is tipped so that the sperm mix with the eggs. A second reading 10 minutes later gives a measurement covering the first 10 minute period after fertilization. In this way the sperm are kept in the bulb only 10 minutes instead of throughout the period of measurement on the unfertilized egg. One such experiment was performed with 40 per cent fertilization.

A Difficulty Peculiar to Nereis

A suitable speed of manometer shaking which did not damage the eggs and yet maintained gas equilibrium between the sea water and the air space above it was found by preliminary trials with unfertilized eggs. A certain rate and amplitude of shake was found to give constant readings. However, following fertilization, the *Nereis* eggs secrete such a quantity of jelly that even a moderate concentration of eggs in the vessel (such as 250 c.mm. of eggs in 4000 c.mm. of sea water) results in a gelation of the whole mass so that the effects of shaking are largely nullified. Gas exchange is limited to the rate of diffusion. The rate of penetration of oxygen into the medium containing the eggs is retarded so as to give an erroneously low measure of consumption. Retention of CO_2 within the jelly may also suppress the respiratory rate of the eggs. This difficulty was encountered in the first experiment. 20 minutes after fertilization a marked decrease in the rate of oxygen consumption to well below the prefertilization rate was observed, after an initial increase. Since this depression period began at about the same time the nuclear sap of the germinal vesicle is liberated into the cytoplasm it presented some points of interest. The vessel contained 250 c.mm. eggs, 100 per cent fertilized, in 4 cc. of sea water, and was found to have gelated to a solid mass, in which however the eggs cleaved perfectly, in spite of suppressed respiration, and at the same time as more dilute controls. Line "d" Fig. 3 shows actual measured rates for this vessel following fertilization. Repetition of the experiment with much reduced volumes of eggs (30-60 mm.³ in 4 cc.) resulted in the

formation of small gelatinous islands or clumps of eggs which floated about freely in the fluid medium. In this medium gas equilibrium could more nearly be maintained. The results of this experiment and of others using dilute egg suspensions showed a much reduced decrease in uptake of O_2 at the time it was observed in the gelled egg mass. In new experiments with concentrated eggs, and gelation, decrease again appeared. The decrease at this time therefore appears to be at least largely an error of measurement due to the suppression of gas exchange by gelation of the medium.

It was necessary therefore to use small quantities of eggs in the experiments in which eggs were fertilized. This of course reduced the effect measured, and therefore the accuracy of measurements in proportion. The accuracy was regained in some cases by extending the time between measurements. In any event the measurements turned out to be accurate enough to show approximately what happens.

RESULTS

The rates of oxygen consumption were calculated in terms of $mm.^3 O_2$ per hour per 10 $mm.^3$ eggs. Following Warburg (3), when KOH is used to absorb CO_2 :

$$X = h \left[\frac{V_g \frac{273}{T} + V_F \alpha}{P_o} \right]$$

where $X = mm.^3 O_2$ consumed, $h =$ change in pressure (observed) in mm. of manometer fluid (Brodie's fluid) at constant volume, $V_g =$ volume in $mm.^3$ of the gas space from the surface of the sea water to the meniscus, $V_F =$ fluid volume in $mm.^3$, $\alpha =$ solubility coefficient of oxygen in sea water (Bunsen's coefficient), $P_o =$ standard pressure in mm. manometer fluid (10,000 mm. Brodie 760 mm. = Hg).

Fifteen measurements were made of the rate of O_2 consumption of unfertilized eggs, each measurement consisting of the average of consecutive readings (usually 10 minutes apart) for between 40 minutes and an hour. The results are given in Table I.

The number of useful experiments measuring the change following fertilization is not so great, as a number of the cases listed in Table I are fertilized controls, or in the case of the earlier experiments with larger volumes of eggs were of no value after fertilization because of excessive gelation.

The results of seven measurements of the rate before and after fertilization are included in Table II. The individual detailed results of two of these are plotted below as samples (Figs. 1 and 2). Fig. 1 shows the actual readings converted to absolute units of one of the best experiments. More than 95 per cent of the eggs fertilized. Unfortunately the simultaneous unfertilized control was rendered useless because of a meniscus of sea water which splashed up over the

TABLE I
Consumption of Oxygen by Eggs of Nereis
Unfertilized Eggs

Experiment	Vol eggs	Vol sea water	Mm. ³ O ₂ per hour per 10 mm. ³ eggs
	mm. ³	cc.	
1	200	4	1.24
2	222	4	1.07
3	250	4	1.35
4	63	4	0.85
5	61	4	0.89
6	58	2	1.50
7	58	2	1.30
8	149	2	1.34
9	150	2	1.10
10	150	2	1.04
11	155	2	0.82
12	35	4	0.91
13	34	4	0.76
14	35	4	1.01
15	31	4	1.10
Average.....			1.10

mouth of the manometer. There is every reason to suppose that it is a reliable experiment however, as the conditions were the same as in other good experiments. The egg concentration is small, 31 mm.³ eggs in 4000 mm.³ sea water, so that suppression of gas exchange by gelation is not great. Fig. 2 represents the results of an experiment with a simultaneous unfertilized control. The period of most of the measurements in this case has been extended to 20 minutes. 100 per cent of the eggs fertilized. There were 35 mm.³ in 4000 mm.³ sea

formation of small gelatinous islands or clumps of eggs which floated about freely in the fluid medium. In this medium gas equilibrium could more nearly be maintained. The results of this experiment and of others using dilute egg suspensions showed a much reduced decrease in uptake of O_2 at the time it was observed in the gelated egg mass. In new experiments with concentrated eggs, and gelation, decrease again appeared. The decrease at this time therefore appears to be at least largely an error of measurement due to the suppression of gas exchange by gelation of the medium.

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7	58	2	1.30
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9	150	2	1.10
10	150	2	1.04
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RATE OF OXYGEN CONSUMPTION BY EGGS. III

31 Mm^3 eggs in 4000 Mm^3 water. - 95% Fertilisation.

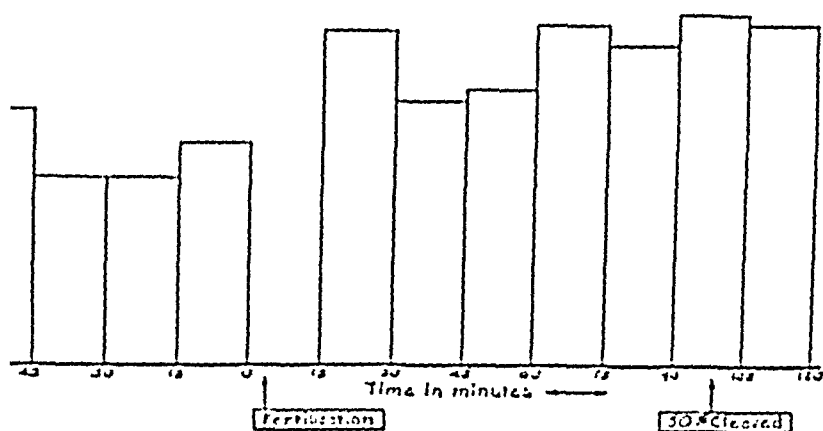


FIG. 1

35 Mm^3 eggs in 4000 Mm^3 seawater - 100% Fertilisation.

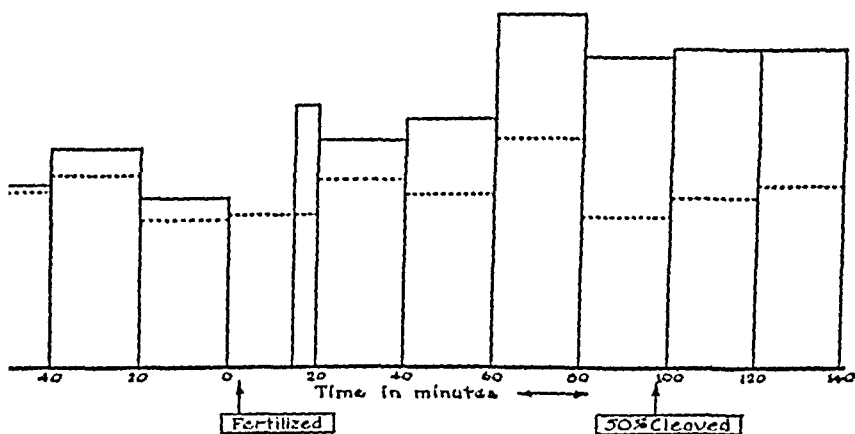


FIG. 2

This experiment shows a relatively less increase in the first following fertilization than most of the experiments. The un-
ed control (dotted line) runs at a rate slightly below average.

The general relationships are typical, however. One reading covering the period 15-20 minutes after fertilization shows a slightly greater rate at this time than just following. This phenomenon was found in all cases.

One experiment (Table II, Exp. 3) was designed for a special purpose. The insemination was performed in the way described in the last paragraph of the section "*Fertilizing the eggs.*" The eggs and sperm were mixed by tipping the manometer, and a measurement was made covering the first 10 minute period following fertilization. In the other experiments no measurement was obtained over this period. 40 per cent fertilization resulted. A high concentration of eggs was used to increase the accuracy of measurement at this period (145

TABLE II

Experiment	Vol. eggs	Vol. sea water	Post-fertilization period average	Fertilization	Rate of respiration of fertilized eggs as per cent of unfertilized
	<i>mm.³</i>	<i>cc.</i>	<i>min.</i>	<i>per cent</i>	<i>per cent</i>
1	63	$\frac{1}{2}$	10-40	100	141
2	61	$\frac{1}{2}$	10-40	95	140
3	145	2	0-40	40	116
4	125	2	10-40	45	136
5	35	$\frac{1}{2}$	15-40	100	125
6	31	$\frac{1}{2}$	10-40	95+	142
7	?	$\frac{1}{2}$	20-40	90+	127

mm.³ in 3000 mm.³ sea water). The suppression due to gelation had previously been found to become effective mostly after this period. It was therefore advisable for the purpose to concentrate the eggs and accept the subsequent suppression. The rate during the first 10 minute period following fertilization was higher than during the second 10 minute period. The suppressing effect of gelation begins to be effective during the end of this period. The rate of the third 10 minute period was below the prefertilization rate. Due to the suppression the results of the experiment are of little value except for the period immediately following fertilization. The first 10 minute period is at high rate. The rise in respiratory rate apparently takes place very soon after fertilization, probably immediately.

The Depression Which Follows Soon after Fertilization

The solid line "Curve *c*" in Fig. 3 illustrates a phenomenon observed in all seven experiments: a gradual decline in rate of respiration following the early post-fertilization measurements until about the period 30-40 minutes after fertilization, after which the rate continues to rise until the end of the experiments. The only visible event taking place in the egg at the time of the decline is the breakdown of the germinal vesicle with the liberation of the nuclear sap at about 18 or 19 minutes after fertilization. This may possibly have something to do with the drop, but there is no good reason to believe so. The beginning of the decline precedes this event in the cases in which the time intervals of measurement afford information on this point. It has been noted earlier that high concentrations of eggs, by virtue of the jelly which they secrete, cause a firm gelation of the sea water, and a marked depression in respiration at about this same time. Line "*d*" Fig. 3 shows post-fertilization measurements in an experiment with concentrated eggs (250 mm.³ eggs in 4000 mm.³ sea water) and with 100 per cent fertilization. It seems probable that the depression as indicated in Fig. 3 "Curve *c*" represents this same effect, reduced in magnitude in vessels containing fewer eggs, but not entirely eliminated. If this is so, the observed values (Fig. 3 "Block *B*" and "Curve *c*") are probably somewhat too low, and the dotted "Curve *e*" Fig. 3 may represent more correct values. This would mean a rate for the first 40 minutes, as well as the first 10 or 20 minutes after fertilization, of about 145 per cent of the prefertilization rate, instead of 135 per cent which is represented by "Block *B*."

SUMMARY AND CONCLUSIONS

1. The rate of consumption of oxygen by unfertilized *Nercis* eggs is about 1.1 mm.³ O₂ per hour per 10 mm.³ eggs.
2. Following fertilization the rate increases to 135 per cent or 145 per cent of the prefertilization rate, and continues to increase for 2 hours (the duration of the measurements).

I am much indebted to Professor W. J. Crozier, under whose direction this work was done, for valuable advice and criticism.

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ANALYSIS OF THE GEOTROPIC ORIENTATION OF YOUNG RATS. III

PART 1

BY W. J. CROZIER AND G. PINCUS

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(Accepted for publication, August 25, 1931)

I

Two preceding papers (Crozier and Pincus, 1929-30, *a, b*) have described the first portion of an attempt to explore the functional basis for quantitative relations between the average extent of upward orientation by young rats creeping geotropically upon an inclined surface, and the slope of the surface. A fundamental question has to do with the "reality" of equations purporting to describe the connection between the mean angle (θ) of oriented path on the surface, during steady progression, and the inclination (α) of the substratum. We have proposed to regard the treatment of this specific problem as illustrating a procedure which has rather general implications (*cf.* Crozier, 1929; Pincus and Crozier, 1929), because in an important respect it seeks to deal concretely with the otherwise troublesome matter of variation of response or performance,—and indeed to make use of it in such a way that the observations provide an automatic "internal" check upon the significance of their mean values.

In certain genetically stabilized lines of rats it was found that for each such line the reproducible relationship between the angle of oriented progression on a sloping surface (θ) and the inclination of the surface (α) could be expressed in terms of convenient quasi-rational coordinates providing graphs sensibly rectilinear, but that the slopes and axis intercepts of these graphs differed in the several races. It was emphasized that the constants in the equations for such relationships should provide the *kinds* of heritable, dimensionally significant, units with which an analytical system of genetics must ultimately deal if the mode of action (the "nature") of genes is to be discussed.

Such units should be in terms functionally meaningful, and it is important that the methods leading to their expression be such as to permit their establishment with single individuals. The demonstration of the hereditary transmission of the basis of such slope-constants, on the other hand, affords proof that the differences in question between the stocks involved are adequately portrayed in the representation chosen for them (*cf.* Crozier, 1929; Pincus and Crozier, 1929), and are to this degree "real" (*cf.* also: Pincus, 1930-31); and the quantitative representation used is consequently given a totally independent justification.

Preliminary demonstration of this sort has been given for two inbred strains, *K* and *A* of our previous accounts, for which the slopes of the graph $\cos \theta$ vs. $\sin \alpha$ differ sharply, the lines crossing one another (Crozier and Pincus, 1927-28; 1928-29; 1929-30, *a, b*). Several consequences of the fact that the $\cos \theta$ vs. $\sin \alpha$ graph is really made up of three distinct sections have been discussed (Crozier and Pincus, 1929-30, *a, b*). By somewhat fortunate accident these particular graphs were sufficiently rectilinear throughout in the pure lines with which the experiment started, although the analysis is not really dependent upon this; it is easily shown that they need not be simple straight lines in every case; there is thus provided, in fact a type-instance for the experimental dissociation of certain kinds of "multiple factor" effects (*cf.* Crozier and Pincus, 1929-30, *b*; and a subsequent paper).¹

The alternative mode of difference among these lines of rats was clearly indicated in the comparison of our races *A* and *B* (Crozier and Pincus, 1929-30 *a*); for these the slope factor ($\Delta \cos \theta / \Delta \sin \alpha$) was roughly identical, but the ordinate intercepts of the graphs quite unlike. Other instances of this sort have been found. It was recognized as important that the breeding test be applied to these strains, and search made for segregation of factors involved in the difference. Our expectation might be that any genetic segregation found would be of a

¹ Another method of establishing the difference between two such lines might be through the equilibration of phototropic and geotropic orientations (Crozier and Pincus, 1926-27, *b*; 1926-27, *c*). This would not be difficult if the photic excitabilities of the two stocks were not too different; but the *A* rats are red-eyed, and constancy of phototropic behavior is not obtainable under the conditions necessary for the test.

sort quite different from that recognized in the case of $A \times K$, and might conceivably even turn out to be a "single" complex or factor. For A vs. K we were able to show, consistently with all the available evidence and with the variability phenomena apparent in the measurements, that it is as if three distinct groups of tension receptors with randomly distributed excitation-thresholds in each group were implicated in gravireception, the three groups having successively higher modal thresholds, and being independently represented by genetic factors responsible for a "large" or a "small" assemblage of the receptors in each of the groups; and that the "large" and the "small" conditions are alleomorphic. The relevant genetic differences between A and K , on this basis, concern chiefly the numbers of sense organs with diverse thresholds for excitation, in the apparently tripartite population of gravitationally excited tension-receptors. It is highly unlikely that any genetically demonstrable differences between lines A and B will be of exactly this sort. This question is now being examined.

Before entering upon this aspect of the matter, however, it is necessary to secure further information about the geotropic behavior of the A and B lines. The present paper is occupied with this. We desired particularly to examine line A with respect to (1) reproducibility of the magnitudes of θ , (2) the constancy of the "variability function," and (3) the influence of added loads in the quantitative distortion of the θ vs. α curve (*cf.*, for line K , Crozier and Pincus, 1929-30, *b*). We give also certain data bearing upon the nature of variation of performance and upon criteria for adequacy of the observational methods. The points of special moment for any such inquiry concern: *reproducibility* of data; *rational formulation* tested by the occurrence of qualitatively identical transformations of the relation between performance and conditions when its quantitative expression differs in diverse instances (*i.e.*, races), under the same kind of experimental treatment; and the quantitative evaluation of the *variability* of the measurements—here defined (Crozier, 1929) as the measured dependence of the dispersions of the measurements upon change of the controlling experimental condition.

II

Experiments made to determine with lines A and B the limits of quantitative reproducibility of the relations between orientation-

angle θ and inclination α of surface are summarized in Tables I and II respectively; additional data concerning B are contained in Table III.

Several points of technic require comment. A more convenient method of recording trails has been employed in experiments subsequent to those of our preceding paper (Crozier and Pincus, 1929-30, *a, b*). A rigid surface for creeping

TABLE I

Mean orientation-angles ($\bar{\theta}$) for young rats of race A , as function of the inclination of the surface (α), in five series of experiments. Series I and II, already published (Crozier and Pincus, 1929-30, *a*), are introduced for comparison (see text); n = number of observations on each individual, N = number of individuals; age = 13-14 days; temperature = $20.2^\circ \pm 1.0^\circ$ throughout. (The subscripts to A indicate the numbers of the close-inbred generations.)

α , degrees	θ , degrees					
	I (A_{10}) $n = 9, 2$ $N = 12$	II (A_{12}) $n = 15$ $N = 5$	III (A_{13}) $n = 15$ $N = 4$	IV (A_{13}) $n = 20$ $N = 4$	IV ₂ (A_{13}) $n = 20$ $N = 1$	V (A_{13t}) $n = 20$ $N = 3$
20	51.88 \pm 0.23	52.81 \pm 1.40		53.43 \pm 1.03	52.80 \pm 2.06	
25	56.97 \pm 0.21	58.27 \pm 1.40	56.55 \pm 1.19	58.23 \pm 1.14		
30	61.39 \pm 0.18	59.28 \pm 1.26	60.04 \pm 1.18	61.03 \pm 0.847		
35	63.19 \pm 0.16	63.05 \pm 1.02		63.84 \pm 0.831	61.60 \pm 1.38	
40			66.90 \pm 0.915			69.51 \pm 0.874
45	71.24 \pm 0.14	71.44 \pm 1.05		72.57 \pm 0.684	74.95 \pm 1.43	
55	77.69 \pm 0.16	78.52 \pm 1.02		77.11 \pm 0.584	77.95 \pm 1.65	
60			77.21 \pm 0.682			
70	80.17 \pm 0.11	79.54 \pm 0.730	78.80 \pm 0.519	80.44 \pm 0.621	81.33 \pm 1.17	

is provided which measures 122 \times 92 cm.; this is covered with close-meshed wire screening, attached to battens adjustable by bolts in slots which permit a taut, flat spread of the screen in contact with the sheet-rock bed of the plane. At the left side this surface is extended by a plane of equal size, not equipped with screen. A large pantograph is anchored to the lower left-hand corner of the second surface. The pantograph is of such dimensions, and is so adjusted, that it permits the recording of paths (scale reduced to 4:1) anywhere on the creeping-plane without

TABLE II

Mean orientation-angles (θ) for young rats of race *B*. Series I, introduced for comparison, is taken from Crozier and Pincus, 1929-30, *a*. Series II rats were a little below normal weight (3 ♂♂, 1 ♀; 14.9 gm.); the temperature was $22.6^\circ \pm 1.4^\circ$.

α , degrees	θ , degrees	
	I B ₁₉ $n = 10$ $N = 15$	II B ₁₉ $n = 20$ $N = 4$
15	55.05 \pm 1.65	55.34 \pm 1.29
20	60.52 \pm 1.34	60.63 \pm 1.07
25		64.40 \pm 0.825
30	68.24 \pm 1.22	68.60 \pm 0.696
35	72.16 \pm 0.85	72.77 \pm 0.776
40		75.90 \pm 0.840
45	77.26 \pm 0.78	76.99 \pm 0.611
55	82.05 \pm 0.51	81.81 \pm 0.591
70	83.98 \pm 0.53	83.58 \pm 0.356

TABLE III

Mean angles of geotropic orientation of rats of line *B'*, collateral-line descendants of line *B* (see text).

α , degrees	θ , degrees	
	I B' ₁₉ $n = 20$ $N = 3$	
20	61.32 \pm 1.04	
25	66.06 \pm 1.08	
35	71.81 \pm 0.885	
45	78.78 \pm 0.637	
55	82.54 \pm 0.466	
70	85.54 \pm 0.336	

distortion. Typical trails are reproduced in Fig. 1. The free point of the pantograph is held immediately above the center of the rat's back. The sliding contact travels in a noiseless, lubricated path. Subsequent measurement from such

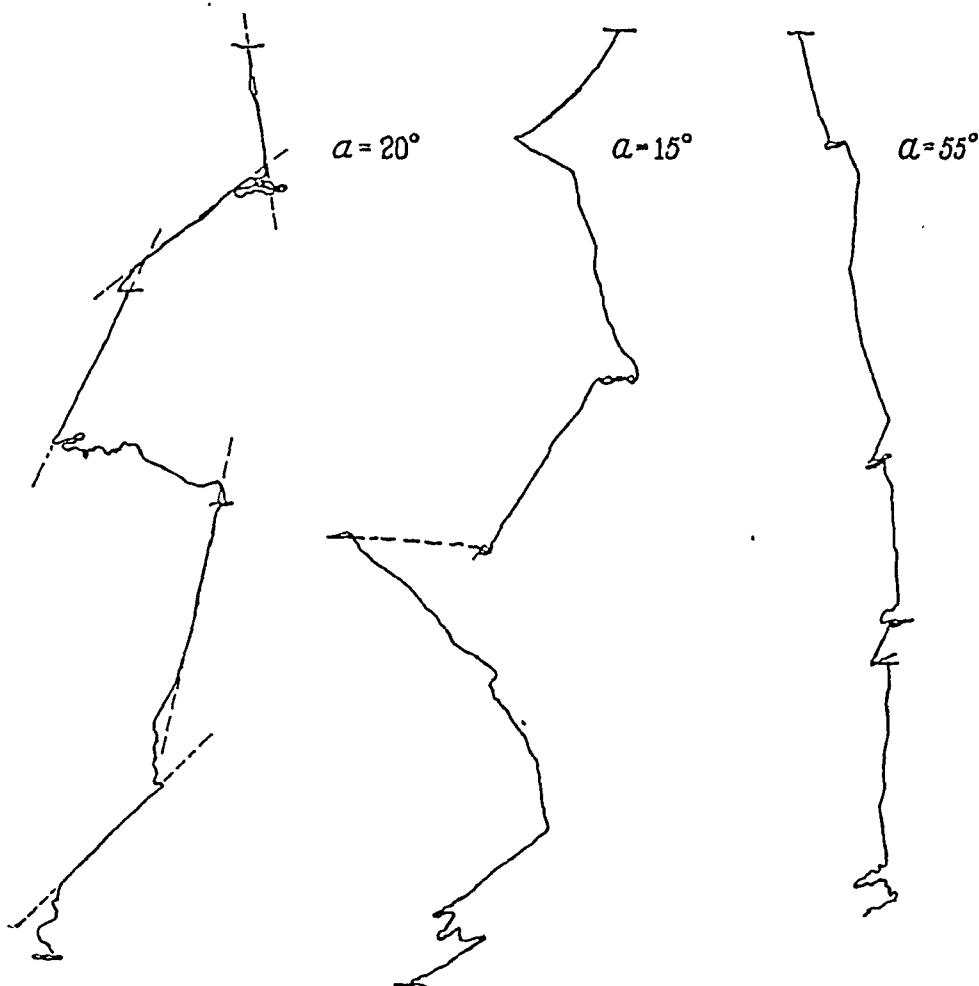


FIG. 1. Trails as recorded by the pantograph method described in the text. Scale = 1:8 after reduction. Conventional marks are interpolated during a run to indicate pauses and re-orientations. One trail shows the way in which angles of orientation are measured. Inclination of substratum = α .

trails is a relatively easy matter. The angle θ is read to the nearest degree of arc on a protractor of 5 cm. radius.

The whole plane surface is so braced and counterpoised that it is inclinable at any desired angle by the smooth action of a heavy worm-gear near the lower margin, controlled by a wheel. A large brass protractor at one side, fitted with a

plumb-line, measures the slope. The slope may be altered while a rat is creeping on the surface, which permits eliminating any immediate effect of handling (*cf. e.g.* Crozier and Stier, 1927-28); this we have found unnecessary, however, for routine experiments. We are under obligation to Dr. Morgan Upton for assistance in the

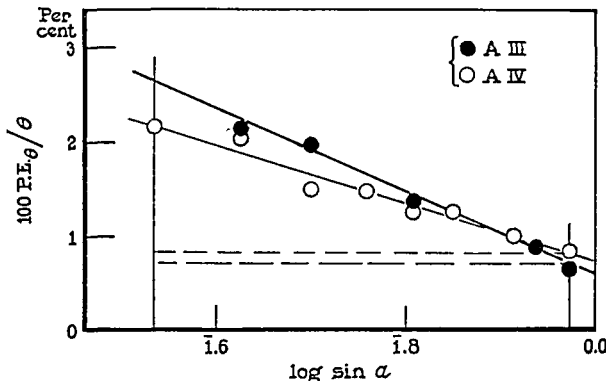


FIG. 2. Variability plots for two series of measurements, (1) by "hand recording" (A III) and (2) by pantograph (A IV'); the modifiable variation in performance (*i.e.*, the *variability*) is sensibly identical; the "residual variation," measured in each case by the area under the dashed line, and which includes errors of recording and of estimation, happens to be slightly (although not significantly) less in the series by "hand recording;" see text.

Series A IV' includes individuals 1, 2, 3 of Series A IV, these being chosen to give $\Sigma n = 60$ for comparison with A III where $n = 15$ and $\Sigma n = 60$. The areas under the dashed lines are in the ratio A III:A IV':0.65:0.83; but P.E._θ is determined not only by Σn but by N , the number of individuals in the set; to make the two areas comparable that for A III must be multiplied by $\sqrt{\frac{15}{20}}$, when it becomes 0.57 and the lesser residual variation in A III is still less than in A IV'. (The difference is not much affected if Series A IV is used for comparison. In this case, with both n and N diverse in the two sets, the corrected unmodified variations are in the ratio A III:A IV::1.58:1.56,—clearly identical.)

The *modifiable variations (variability numbers)*, in the two series are also substantially identical. Computed as described in the text (p. 218), these are, for A III, V.N. = 3.22; for A IV', 3.11; for A IV, 2.91.

construction of these inclined planes, also used in his experiments with rats and guinea pigs (Upton, in press; Upton and Stavsky, in press).

It is of interest to compare the data secured by this method with that previously obtained by a sketch method of recording upon coordinate paper (Crozier and Pincus, 1929-30, *a*; and earlier publications there cited). The methods of handling the animals, with attention to temperature, illumination, ventilation of the dark-room, exhaustion, feeding, and the like, the age of the animals, and the size of the nursing litter, were as nearly alike throughout as possible. A test of this kind is given by Series *A* III and *A* IV, done in succession. The mean θ 's from the two series, one by "hand recording," the other by pantograph, are sensibly identical.

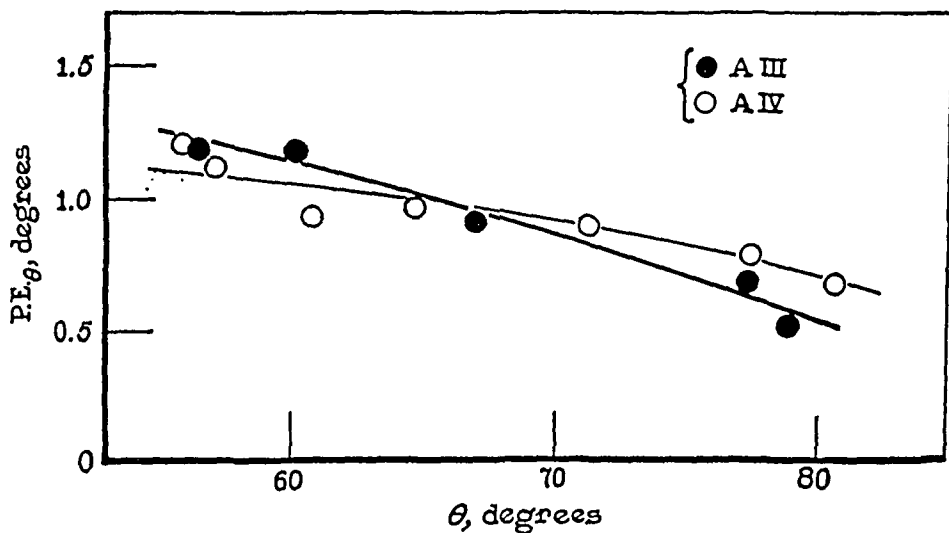


FIG. 3. P.E. $_{\theta}$ is plotted against θ , for Series *A* III and *A* IV'. The upper limit of working is near $\theta = 80^{\circ}$; the non-reducible variation is less in *A* III, the modifiable variation greater, than in *A* IV; this accords with the result given in Fig. 2. See text.

We might expect, however, some difference in the *variation* of the measured orientations. The total variation of performance, however, is divisible into two portions (Crozier, 1929), one modifiable according to the magnitude of the variable controlling the response ($\sin \alpha$), the other not so modifiable and including the errors of recording and estimating θ . We find, in fact, that for our contrasted series of measurements the "index of variability" is to all intents and purposes identical (Fig. 2); the residual variations, given by the areas under the dashed lines, include errors of recording and of estimation, independent of α , as well as the unmodifiable fluctuations of θ , and do not differ greatly enough for significance—although the "accuracy" here seems to be slightly in favor of the non-pantograph series! Two series by the same mode of recording are likely to show even larger differences,

however. Tests of this kind permit the assertion that series of estimations by the two methods of observation are legitimately comparable. Since it might be surmised that errors of observation (particularly "by hand") might in one way or another be a function of θ , the comparisons may be made in another way (Fig. 3). Similar tests have been made with other sets. A more searching check, however, which allows for the effects of intrinsic differences in the total variation encountered in each case, is given by the *areas* under the variability graphs (Fig. 2, etc.). These areas are such that for *A* III the "unmodifiable variation" is 0.669 units (corrected for *n* and *N*), and 62.1 per cent of the total measured variation is of the controllable type; for *A* IV the unmodifiable variation is 0.678 units, the controllable variation being 51 per cent of the total. As indicated later on (Table V), the difference is entirely within the limits of scatter for determinations of these quantities with different litters by the same method of observation. The result as regards the relative reliability of *A* III and *A* IV is as in the previous comparison.

A parallel test of the two methods of recording is permitted by the data from litters *B* I and *B* II. Here, the total uncontrolled variations are respectively 0.413 and 0.326 units, the controllable variations being 70.0 and 78.3 per cent of the corresponding totals. The fact that in these series of *A* and *B* rats the total "residual variation," *i.e.*, the unmodifiable relative variation of θ , is characteristic of the stock (under the conditions used) is later discussed with other findings; its interest at the moment is in its indication that even the uncontrolled fraction of scatter of θ 's is not primarily a matter of any "error of observation," by either method of recording.

The fact that the *total variation* is statistically almost if not quite constant and characteristic for each race (*A*, *B*, *K*), under comparable conditions, and the percentage of the total which is modifiable, disposes completely of the consideration that the judgment of the observer as to when the animal is really oriented, and its path hence suitable for pertinent measurement, may bias the outcome in some fashion. In point of fact there is no difficulty in such judgments, after practice, as proved by the concurrence of findings by two observers. But we consider it important that objective criteria of the sort here used should be developed and tested. They serve as a useful illustration of the necessity for definition of units of measurement in functional terms, and of the desirability of experimentally dissecting the raw standard deviations of the mean magnitudes of measurements. We are entirely alive to the fact that refinements of the statistical methods are conceivable in several directions, but the practical outcome indicates that (in the cases which here concern us) there has been effected a reasonable and adequate compromise with certain purely experimental limitations.

III

The assembled mean θ 's for line *A* are plotted in Fig. 4 (Table I), against $\log \sin \alpha$, and for line *B* (Tables II, III) in Fig. 5. It is apparent that the quantitative correspondence between the respective mem-

bers of each set is remarkably close. The criterion of *reproducibility* appears to be met in these measurements: with line *A*, observations with litters from generations 10 to 19 of closest inbreeding yield concordant relations between θ and α ; for line *B*, observations with litters from generations 10 to 18 yield similarly consistent relationships. In the case of line *B* a supplementary test is possible by means of descendants in a collateral line of this stock.

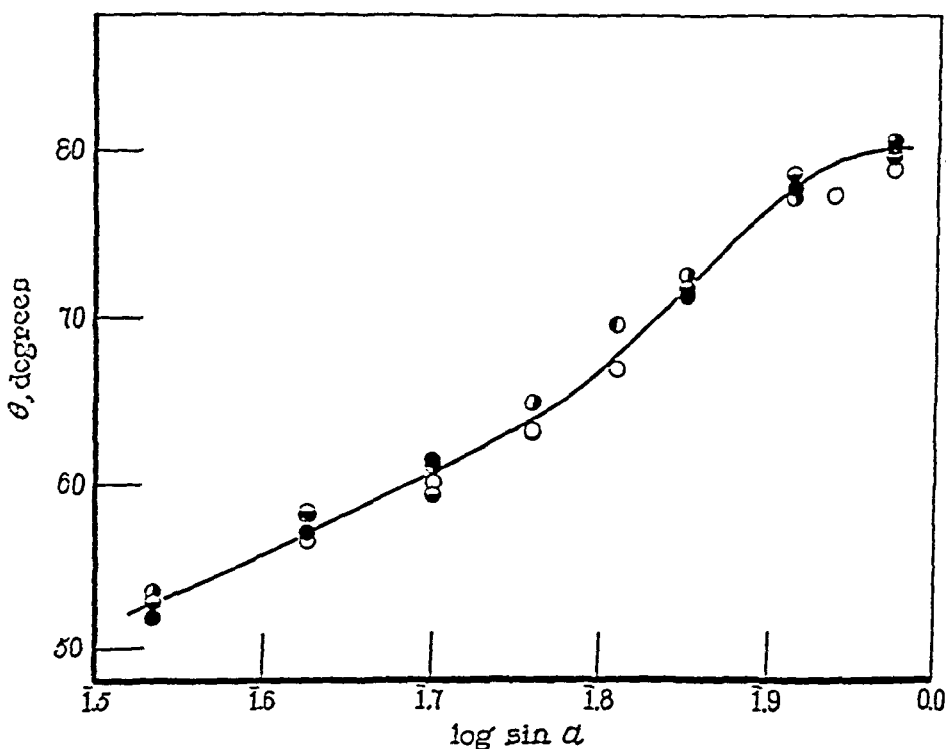


FIG. 4. Mean θ 's for line *A*, as function of $\log \sin \alpha$, from several series of measurements (data in Table I). P.E.'s for the different sets overlap.

This collateral line (*B'*) is of the fifteenth brother-sister generation, and since 1927 has been bred independently of the *B* animals of our first two series by Dr. H. W. Feldman of the University of Michigan.² The animals of the Feldman stock are genetically red-eyed, hooded albinos, and this particular line was separated from our stock at

² We are very much indebted to Dr. Feldman for supplying us with these animals.

about the ninth brother-sister generation. Our B animals lack albinism, but closely resemble those of the Feldman stock in general anatomical proportions. Observations with individuals of this line (" B' ") are given in Table III, also plotted in Fig. 5. The mean θ 's are on the whole rather a little higher with B' than with either of the B litters, but this is not really significant; the standard deviations for B and B' overlap.

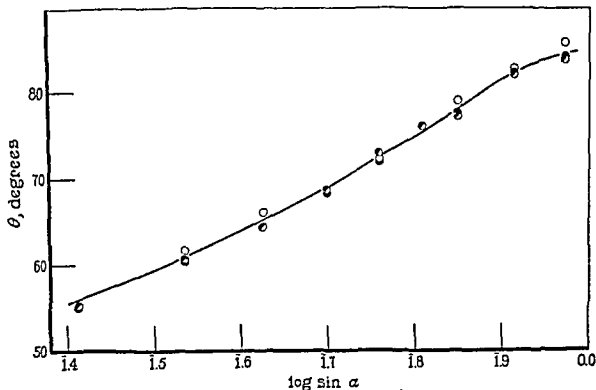


FIG. 5. Mean θ 's for line B , as function of $\log \sin \alpha$, from several series of measurements (data in Table II). Observations on B' (open circlets) are included. The P.E.'s overlap.

A second set of observations on line B' has been made since this paper was completed. The result is of interest because it gives further material checking the B curve, and because the mean θ 's are consistently identical with those for B litters, thus justifying our decision that the *slightly* higher θ -values for B' given in Table III did not depart significantly from the values given in Table II; they demonstrate, also, that the identity of B and B' litters extends to the value of mean θ at $\alpha = 15^\circ$. A tabulation of the additional data is included here as a matter of record:

Line B', Second Series; N = 3, n = 20

α , degrees	15	20	25	35	45	55	70
θ , degrees	55.7 ± 1.36	61.0 ± 1.10	64.6 ± 1.03	72.6 ± 0.686	77.9 ± 0.710	82.0 ± 0.433	83.4 ± 0.351

The variability number is 3.37; $\Delta P.E._\theta / \Delta \sin \alpha = 0.61$; the proportionate relative variation = 71.9 *per cent*; these values agree satisfactorily with those previously found (Tables IV, V) for *B* and *B'* individuals.

The important indication is the high degree of stability of the geotropic performance in "pure lines" of rats. This is equally apparent in the *variability* of orientation, which we have now to examine.

IV

The reproducibility of the relationship between α and θ for the several inbred lines finds its counterpart in the reproducibility of the measures of variation of θ . The theory of the estimation of *variability* (Crozier, 1929; Crozier and Pincus, 1926-27, *a*; 1929-30, *a*) made use of the fact that in the case of these geotropic responses the relative variation of mean θ , in any given set of measurements, should depend upon the gravitationally effective vector in the same way as the measure of orientation (θ) itself. The plots of θ *vs.* $\log \sin \alpha$ are sufficiently rectilinear to permit using the graph of σ_θ / θ *vs.* $\log \sin \alpha$ as a convenient test of this, but it is for some purposes more suitable to plot σ_θ / θ against θ . Here σ_θ is the standard deviation of *mean* θ ; we have systematically used $P.E._\theta$ rather than σ_θ , as being more convenient, but this in no way affects the use of the final expressions we seek. The number of observations is of course identical throughout each set.

From such graphs an index of the variation of geotropic orientation is computed as a function of the exciting variable; this index may be termed the "variability number," and we shall so refer to it. For practical reasons, we have secured observations by measurement of twenty angles of orientation at each of a number of slopes of surface (6 to 8 or 9) with each individual of a small litter, 13 to 14 days after birth. It is not practicable to take many more observations without

introducing irrelevant variations. We have therefore followed the practice, in previous accounts, of averaging the orientation-angles at each slope, getting in this way a mean θ for the litter or group. With other litters different slopes of surface may then be used to give more closely distributed points on the plotted curve. The essential procedure is to obtain with each animal, in any one set of measurements, the same number of observations at each magnitude of the independent variable; when this n_i is slightly lower or higher in any one instance, correction is applicable provided n_i is so large that $\Sigma v \propto n_i$. The close agreement of the results among single individuals makes the averaging process legitimate; the rare exceptions to this require individual consideration (*vide infra*).

In the plots of relative variation against $\log \sin \alpha$ (*cf.* Figs. 2, 8, etc.) the observations extend from a threshold value of α , which in these measurements can be given as $\alpha = 15^\circ$ for *B*, 20° for *A*, but cannot easily be located more exactly, up to $\alpha = 70^\circ$ or thereabouts. Above this slope, θ_{mean} tends to be constant, σ_θ to rise, owing to the occurrence of a tendency to slipping (*cf.* Crozier and Oxnard, 1927-28). The area under the graph, between these limits, comprises two parts: that below the dashed line (Fig. 2, etc.), which is the total measured variation not modifiable according to the magnitude of the effective gravitational component; and that above the dashed line, which is thus modifiable. That the line of division between these areas is parallel to the $\log \sin \alpha$ axis is proved by comparing the graphs for series in which the numbers of individuals, or of observations taken on each individual, or both, are different (Crozier and Pincus, 1929-30, *a*). Such comparisons justify experimentally a procedure of computation which has other justifications in addition:

When n observations on each of N individuals are concerned, the N individuals being a sufficiently homogeneous group, so that the dispersion of their means is very slight, the P.E. of the mean of the series of sets may be taken as $[(0.8453\Sigma v)/(\sqrt{n_i(n_i - 1)})] \times 1/\sqrt{n}$, where v is the deviation of one observation from the mean of its set, and $n_i (= Nn)$ is the total number of observations. Then the product $(\sqrt{n/N})$ (Δ P.E.) will change in proportion to the change of $(\Sigma v)/\sqrt{Nn_i(n_i - 1)}$; so that this change, taken as a function of some unit alteration of a

controlling factor, gives the mean change of the mean root-mean-square deviation from the general mean, per individual, per unit change of the independent variable, sensibly independent of n and N , on the assumption that $\sigma \propto \sqrt{N}$. When data from one individual only are concerned, $\Delta P.E._\theta$ is of course taken directly.

If it is desirable to estimate variability as a function of θ , or σ_m as a function of some variable, a similar method is followed. When discontinuities in the relation between θ and $\log \sin \alpha$, for example, are for any reason involved, it is better to consider σ_m (or σ_m/θ) as it depends on θ (*cf.* Upton, 1929-30; Hoagland and Crozier, 1931-32; etc.). It is rather remarkable that plots such as those in Figs. 2, 8, 9, etc., should be so generally rectilinear as they are found to be; one would expect, rather, a fan-wise distribution of points, with sufficient groups of observations, wide at the low- α side since each P.E. is of course subject to a probable error proportional to $1/\sqrt{n}$ and to its own magnitude, and in these series n is constant in each set; in a number of cases this is indeed found (*cf.* Crozier and Pincus, 1929-30, *a*; Figs. 10, 11, 12), and would be looked for if several successive determinations of θ at each of a number of slopes (α) were to be made.

We have been aware that a variability number computed in this fashion must depend to some extent upon the particular technic of handling the animals; but the consistencies apparent in the results demonstrate that it is not an artificial measure; its possible dependence upon experimental conditions is in fact advantageous, since it permits the investigation of the real significance of "experimental conditions" as they affect variation. An instance, although in several respects defective, may be given in illustration: in work by Upton and Stavsky (1932) the orientation of suitably treated *adult* rats of line *K* was measured; a discontinuity is obvious in the θ - $\log \sin \alpha$ curve, but $P.E._\theta/\theta$ vs. θ is continuous and essentially rectilinear (Fig. 6); the variability number computed on this basis, with $\Delta\theta = 20^\circ$, is 0.767 units, distinctly lower than the corresponding index for the young *K* rats (Crozier and Pincus, 1929-30, *a*), which on the same basis (two series) is 2.57, 2.96; we cannot tell, in this instance, whether the adult condition is responsible for the difference, because the fact that the observations with the mature animals were spread over several days would be

expected, of itself, to reduce the proportion of scatter of observed θ 's which is limited by the gravitational vector; the influence of the preliminary treatment of the adult rats is likewise as yet obscure. But a little closer scrutiny permits some of this uncertainty to be removed.

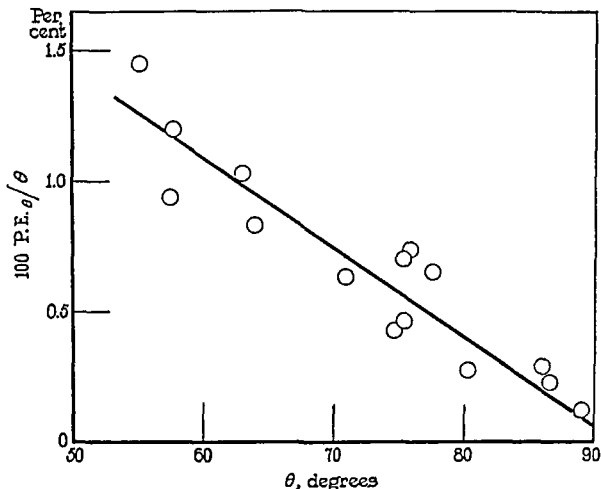


FIG. 6. Data from an experiment by Upton and Stavsky (in press) with *adult* rats of race *K*, made geotropic in behavior by appropriate training (which has no effect on the specific relation of θ to α); the plot of θ against $\log \sin \alpha$ exhibits an abrupt discontinuity in its middle, but the smooth dependence of relative variation on θ continues unaltered; the nature of the scatter of the points is referred to in the text. The variability number computed as $\Delta (100 \text{ P.E.}_\theta / \theta) / (\Delta \theta = 20^\circ)$ is 0.77 when corrected for $n = 10$, $N = 8$. See text.

The total observed variation (Fig. 6), the area under the graph, is 23.1 in the units given by the coordinates; of this amount 19.7 units, or 85.3 per cent is modified according to the magnitude of θ . For our Series *K I* and *K III* the percentages of modifiable variation are respectively 73.0 and 85.3. Even for our Series *II* (Crozier and Pincus, 1928-29;

1929-30, *a*), known to be technically unsatisfactory, the percentage works out at 84.6. The facts, so far as they are available, certainly speak for the "fraction of relative variation which is governable" as a thoroughly *intrinsic* property under "standard" conditions; we shall refer to this percentage as the *proportionate relative variation*. The development of these conceptions is reserved for treatment hereafter.

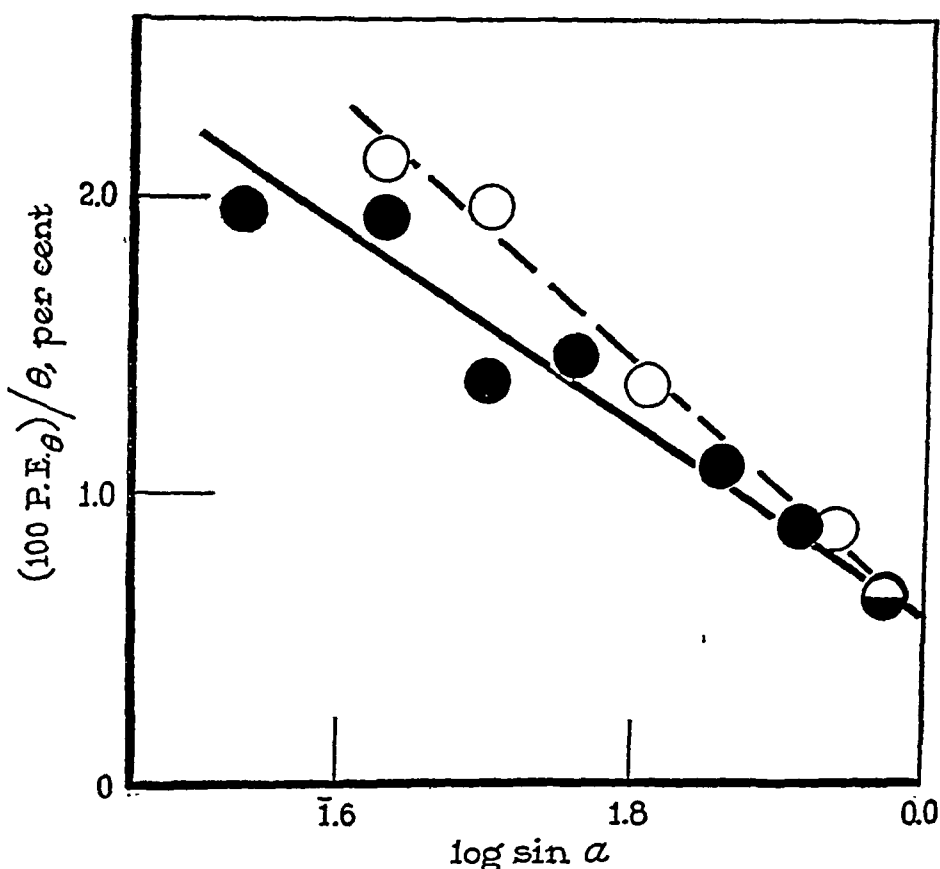


FIG. 7. Relative variation of orientation, line *A*; Series III, open circlets; Series IV, solid circlets.

For line *A* plots of relative variation of orientation are given in Figs. 2 and 7. The *variability numbers* are given in Table IV. The corresponding material for lines *B* and *B'* is set out in Figs. 8, 9, 11, 12, and in Table IV. Within each line the measure of dependence of change of variation upon change of exciting force shows good con-

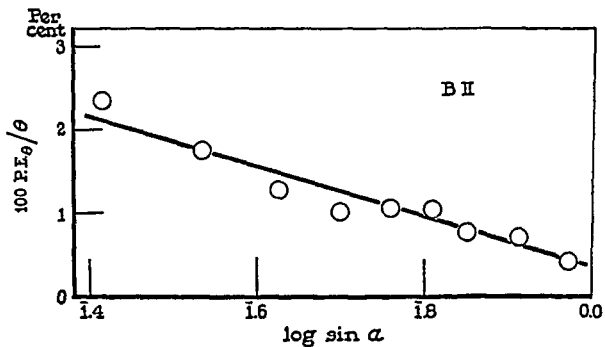


FIG. 8. Relative variation of orientation, Series B II.

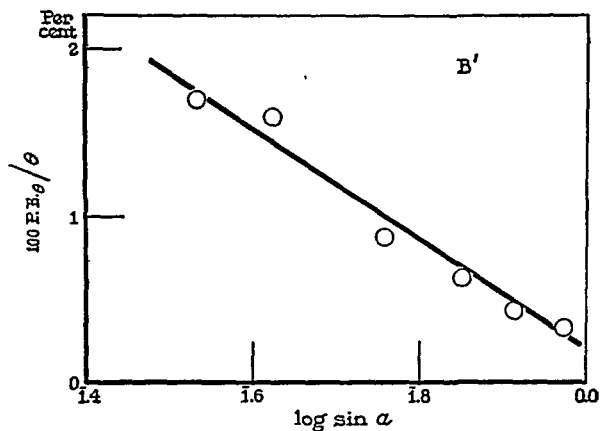


FIG. 9. Relative variation of orientation, Series B' I.

stancy. For B , V.N. appears to be slightly but definitely higher, and with $\Delta P.E._\theta / \Delta \sin \alpha$ also. In comparing the variabilities of lines A , B , account must be taken of the fact that the thresholds for definite orientation are unlike. With race B orientation is always found at $\alpha = 15^\circ$; with race A , at $\alpha = 20^\circ$ but not below this slope. In arbitrary units, correcting as before for N and n , the total modifiable variations with A and B are respectively $56 \pm$ per cent and $74 \pm$ per cent of the respective

TABLE IV

Variability numbers (V.N.) for geotropic orientation of young rats of lines A and B ; N = number of individuals, n = observations on each; computed from $\Delta (P.E._\theta / \theta) / \Delta \log \sin \alpha$.

	Series	N	n	V.N.	
A	I i	6	8.3	2.83	Weighted mean 2.82
	I ii	6	10.0	2.32	
	II	5	15	3.13	
	III	4	15	3.22	
	IV	4	20	2.91	
	IV'	3	20	3.11	
	IVa	1	20	2.2	
B	I	5	10	3.79	Weighted mean 3.32
	II	4	20	2.68	
B'	I	3	20	3.38	

total variations (*cf.* Table V). We shall later have occasion to utilize such figures.

Since the mean θ 's for B' are perhaps a little higher than for B , in the series available, it is instructive to compare the variability as a function of θ . For the separate individuals this is done in Fig. 10. It is obvious that each set is self-consistent. For B' the relative variation is constantly lower than for B ($P.E._\theta$ is as well), but $\Delta(P.E._\theta / \theta) / \Delta \theta$ is a little higher, as the previously calculated V.N.'s have already

indicated by other criteria (Table IV); the total variations (Table V) are such, however, that the percentage which is modifiable is identical in B and B' . We have consequently taken B and B' series together in plotting Fig. 5. Litters in our B line have been difficult to secure and to raise, and B' individuals are somewhat heavier and sturdier at 13 to 14 days after birth. Particularly at $\alpha = 70^\circ$ we have taken these facts (and P.E. $_\theta$) into account in fitting the smooth curve (cf. also Section III).

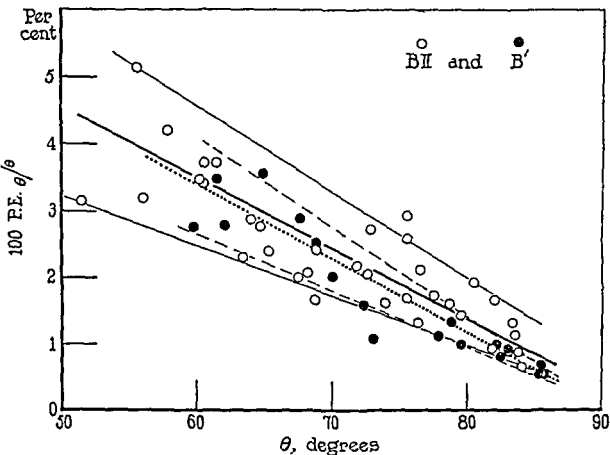


FIG. 10. P.E. $_\theta/\theta$ vs. θ for B and B' . See text. The data for each individual are plotted separately (cf. Fig. 11, Fig. 12).

The total measured variation, in plots of the sort we have been considering, is the area under the line fitting the points, delimited by the ordinates at threshold α and at maximum usable α . For testing consistency of variation in behavior we have employed the "variability number" previously defined, as expressing the manner in which the variation of θ depends upon $\sin \alpha$. For the comparison of diverse races, however, this procedure must be amplified to take into account

correspondence, and the degree of spread is the same. From the method of computation, already outlined, weighted means are obtained by multiplying each determination by N . For example, with line A the extreme variation among individuals is from 44 *per cent* (unusually aberrant individual) to 56 *per cent*. This has practical importance in the classification of individuals in breeding tests.

Two considerations interfere with attempts to deal, as yet, with the absolute magnitudes of the total variations in different litters of the same strain: we do not know, experimentally, how the total variation depends upon such conditions as relative degree of development, conditions of feeding, temperature, humidity, and the like; and we cannot as yet decide if the upper limit of α ($= 70^\circ$) has been precisely enough chosen in all cases, nor can we thus far tell whether the threshold slope (α) varies slightly from litter to litter under the conditions employed. Under these circumstances the agreement in parallel determinations of the percentage of variation which is modifiable must be regarded as rather remarkable. It is clear that if the proportion of variation in $P.E.g/\theta$ which is governable by $\sin \alpha$ is a reproducible property of a race, it should be possible to employ this ratio in locating threshold values of $\sin \alpha$, particularly under different experimental conditions—although this must be checked by tests involving such variables as light, temperature, humidity, and inner conditions of the tested organisms. The moieties of total variation may be estimated from the $\log \sin \alpha$ graphs, using threshold α 's for the lower limits of orientation; or, concordantly, from plots of $P.E.g/\theta$ *vs.* θ , using the lowest orientation θ in each case as the lower limit.

It must be apparent that the notion here employed raises certain difficulties in the way of loose comparisons of variation of performance. Quite apart from the fact that several really separate ideas commonly associate haphazardly under this label (Crozier, 1929), it is apparent that in cases for which the comparison of lines A and B may serve as a model it cannot be said simply that B is "more variable" in geotropic response than A , although the total integrated observed variation is larger, because the threshold for measurable response is lower with B ; if the comparison were to be restricted to identical ranges of $\sin \alpha$ ($\alpha = 20^\circ$ to 70°), B would be a little "less variable." Moreover, the *uncontrolled* variation of B (under the given conditions) is distinctly less than

with A ; but the percentage of the total variation in orientation which is governable according to the intensity of the exciting condition is definitely higher; if this comparison, again, were made over an identical range of $\sin \alpha$, neglecting the lower threshold for B , the relationship would be reversed. The reasons for differences in effective slope-threshold are discussed elsewhere. The findings summarized in Table V demonstrate, however, that the measures of the several aspects of variation are intrinsic, quantitatively reproducible properties of the several distinct homozygous strains tested; this conclusion is substantiated and amplified by results with other material.

ANALYSIS OF THE GEOTROPIC ORIENTATION OF YOUNG RATS. III

PART 2

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V

Thus far, in speaking of variation of performance, we have discussed chiefly the *relative variability* of θ , on the ground that for these cases the precision of the mean of the observations should be limited in the same way as the mean angle θ itself, which analysis permits us to regard as the measure of excitation (Crozier and Pincus, 1929-30, *b*). The direct control of excitation, however, should be proportional to $\sin \alpha$ (careful test shows no effect of weight of individual, or sex, other things equal, so far as θ is concerned). We therefore look for the slope $\Delta\sigma_\theta/\Delta \sin \alpha$ to be constant in each series of measurements. Clearly, a variation index (of slightly different meaning from the one hitherto used) should be computable on this basis also. The plots in Fig. 13, 14, 15 demonstrate that this expectation is satisfactorily met by all the available measurements, including those previously published. For subsequent reference, we may include here results with line *K* as well.

When dealing with the reaction-time or latent period for geotropic response (in snails) it is found that σ_t is directly proportional to the latent period, both being influenced in the same manner by $\sin \alpha$ (Hoagland and Crozier, 1930-31); the case is similar with θ and σ_θ in the gravitational orientation of *Uca* (Kropp and Crozier, 1928-29) and of ants (Barnes, 1929), where $-\Delta\sigma_\theta/\Delta \sin \alpha$ is constant. The extension of this method of analysis to other types of occurrences (Crozier, 1929; Crozier, Stier, and Pincus, 1929; Pincus, 1930-31) is important, because it is obvious that no predisposition of the ob-

server³ could achieve by conspiracy or accident a state of affairs in which the *scatter* of the observations is such that the standard deviations of their means shall obey rational rules (in the present case, 3

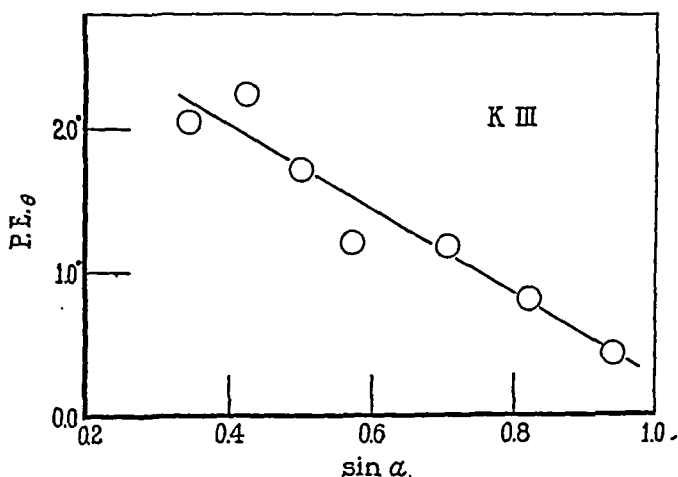


FIG. 13. P.E._θ vs. sin α, in line K (Series III).

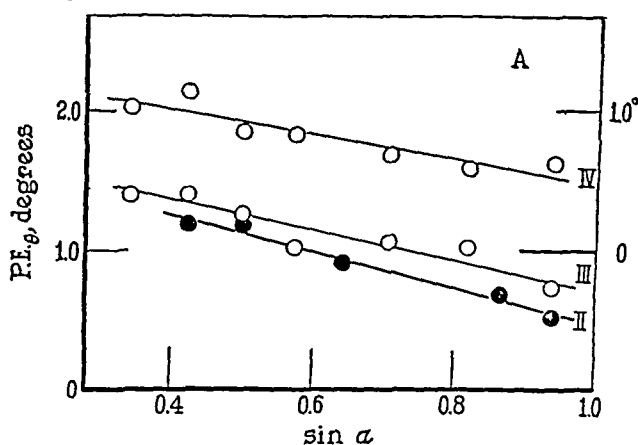


FIG. 14. P.E._θ vs. sin α, in line A.

distinct and specific rules), in some instances not looked for until long after the data were recorded! It has been emphasized (Crozier, 1929) that where tests of this character can be applied it is no longer per-

³ Or rather, of two observers; we have made a practice of dividing the labor of recording, so far as possible. The variability number is independent of the observer when series by each are compared.

missible to speak loosely of "variability of conduct," because the variation of performance is itself lawful.

VI

It is notable that, when several series of observations are available, as with line *A* and in other material at our disposal but not here considered, in which the effect of casual variations of mean θ 's is reduced

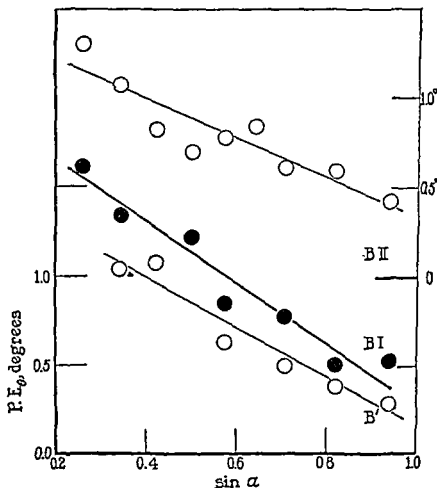


FIG. 15. P.E. _{θ} vs. $\sin \alpha$, in line B.

by having n sufficiently large, the dispersion of the mean θ 's tends conspicuously to be confined, in the plot against $\log \sin \alpha$, to a band with definite marginal delimitations. The "width" of such a band is very nearly constant, if not indeed absolutely so, in the direction parallel to the $\log \sin \alpha$ axis; the indication is that with plotted points of equivalent weight the "width" would be quite constant. This must be a reflection of the "unmodifiable" fraction of the total variation of performance (*i.e.*, independent of $\log \sin \alpha$). It signifies that

to produce an unequivocal increase of mean θ , the inclination of surface (α) must be increased by an amount which in terms of $\sin \alpha$ is a definite fraction of the lowest $\sin \alpha$ which unequivocally invokes the lower θ . Lest it be mistakenly assumed that this is the Weber-Fechner Rule in some sort of disguise, we hasten to point out that a definite fractional increase of $\sin \alpha$ thus leads to a statistically significant step in θ , but that the magnitude of the resulting $\Delta\theta$ is manifestly a function of the magnitude of $\sin \alpha$. Another way of describing this state of affairs is, that the latitude of variation in $\sin \alpha$ over which a statistically constant θ may be expected in a uniform population is a constant fraction of the mean $\sin \alpha$ associated with this orientation-angle. For line *A* this latitude is *ca.* 10 *per cent* of $\sin \alpha$, for mean θ 's of the given weights, and in line *B* also. This type of dispersion imposes definite conditions upon the methods permissible in fitting curves to the observations.

VII

In races *K*, *A*, and in *R. rattus* (Crozier and Pincus, 1926-27) $\Delta \cos \theta / \Delta \sin \alpha$ was sensibly constant. Crosses between *K* and *A* showed that this need not be true in all cases (Crozier and Pincus, 1929-30, *b*). We were inclined to disregard certain indications in the data for line *B* (1929-30, *b*), because we then had at our disposal only one series of measurements. The present material on line *B*, and data on other lines which we shall discuss in later papers, force the recognition in *B* of a state of affairs like that experimentally produced in our crosses *K* \times *A* and in back crosses with these. This has been referred to in the first section of the present paper. We now record the condition in these lines for convenience of reference in our subsequent account of the results of crossing lines *A* and *B* (*cf.* Fig. 16). The *B* line definitely shows a lesser slope ($\Delta \cos \theta / \Delta \sin \alpha$) at the high- α end than at lower α 's. The reason for this becomes apparent in the subsequent discussion. The straightness of the graph up to $\alpha = 48^\circ$ is less clear when the data from several tests is plotted together, since there are slight tendencies for one set to show higher θ 's than another; this cannot be properly taken care of by averaging $\cos \theta$'s. The slope of the line drawn averages the slopes for the individual sets. It should be noted that although a rough derivation of the relation between θ and α has

been suggested in outline, from the standpoint of the theory of the geotropic orientation (Crozier and Pincus, 1926-27), and can be given in more rigorous form, it has not been used save as a method of representation which is for certain purposes convenient (Crozier and Pincus, 1929-30, *a*, *b*); its more exact use requires recognition of the three regions of action of discrete groups of excitation-units. It happens

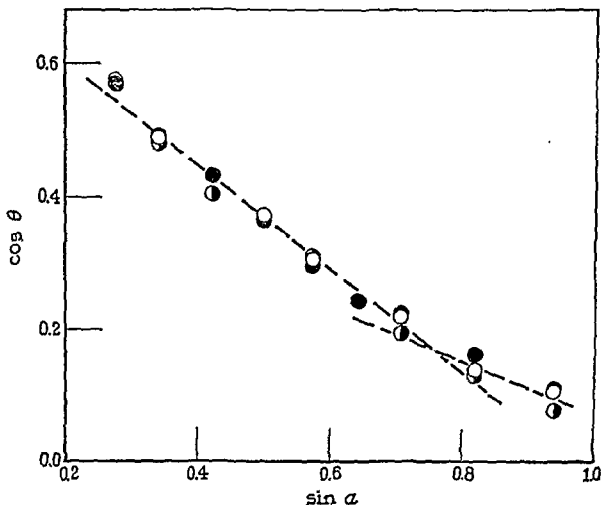


FIG. 16. $\cos \theta$ vs. $\sin \alpha$, line B. See text. (Approximate rectilinearity is observed empirically up to an inclination $\alpha =$ about 48° . The plot really indicates, however, three distinct straight portions, the two below $\alpha = 48^\circ$ being for *B* rats, as for *K*, *A*, etc., practically confluent.)

that for *A* and *K* rats the three regions of the graph have about the same slope. For *B* this is almost true for Groups 1 and 2, but not for Groups 2 and 3.

VIII

The argument leading to the analysis of the curves θ -log $\sin \alpha$ need not be reproduced (*cf.* Crozier and Pincus, 1929-30, *b*); reason has been

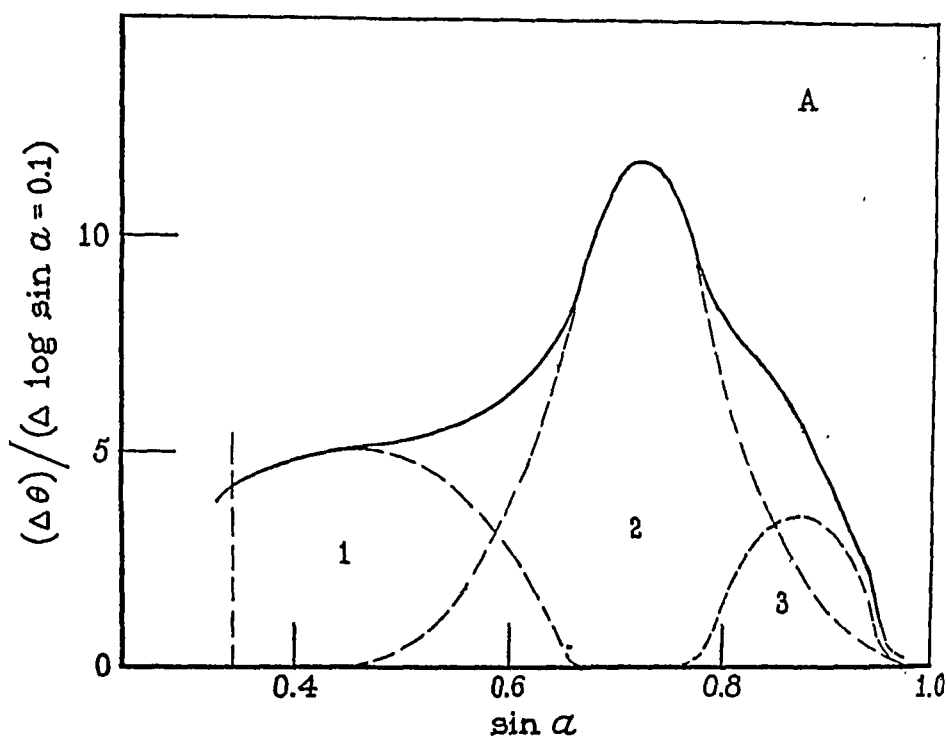


FIG. 17. $(\Delta\theta)/(\Delta \log \sin \alpha)$ as function of $\sin \alpha$, for line A. See text. The graph is resolved into three constituent areas, labelled 1, 2, 3. In terms of the analysis given, these areas represent frequency distributions of thresholds for excitation-units.

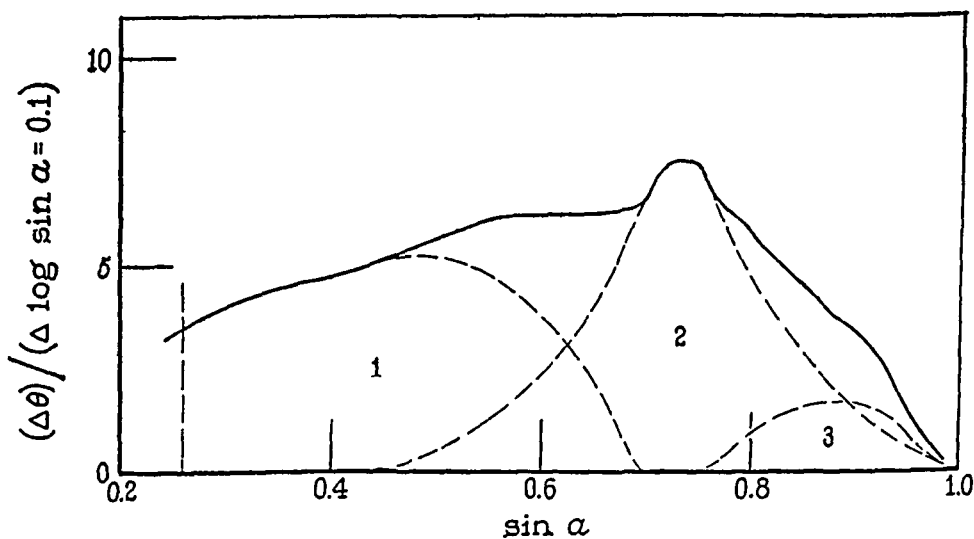


FIG. 18. $(\Delta\theta)/(\Delta \log \sin \alpha)$ vs. $\sin \alpha$, for line B.

given for considering the plotting of $\Delta\theta/\Delta \log \sin \alpha$ against $\sin \alpha$ as providing a picture of the population of receptors concerned in the response in terms of their thresholds for excitation. The limitations of the assumptions employed have been considered (1929-30, *b*). The most searching test of the adequacy of this interpretation has been given by breeding tests, and is amply consistent with it. The differential curves for lines *A* and *B* can now be given more precisely than was possible previously (Figs. 17, 18). These curves do not differ in any material way from those previously published, however. They show that *A* and *B* rats are in a general way similar as regards the forms of the three sub-curves ("groups of receptors") into which the differential curves may be resolved, but that Groups 2 and 3 are definitely smaller in *B* than in *A*, "3" being about one-half the corresponding area in *A*; Group 1, however, is definitely larger in *B*, even when allowance is made for the lower threshold inclination which obtains with *B*. The maxima for the corresponding sub-curves for the two races turn out to fall at almost identical values of $\sin \alpha$. The areas under the 3 sub-curves are, in arbitrary planimeter units on an identical scale:

Race	Areas of "Groups of receptors"			Total
	1	2	3	
<i>A</i>	3.10	6.18	1.12	10.40
<i>B</i>	4.46	4.10	0.65	9.21

It is instructive to compare these areas with the relative variations of response. Including corresponding indices for the *K* line, from data previously published, we have:

Line	Area $\frac{\theta}{(\log \sin \alpha)}$ vs $\sin \alpha$	Total variation (mean)	Proportion of variation modifiable (mean)	Variability number (mean)
			<i>per cent</i>	
<i>K</i>	17.3	2.8	85	5.85
<i>A</i>	10.4	1.3	56	2.82
<i>B</i>	9.20	1.5	72	3.32

Since a larger range of observed θ 's, and of usable $\sin \alpha$'s, tends automatically to increase all these quantities save the variability number, it might be suspected that "number of receptor units," total variation of response, and proportion modifiable according to excitation, should be parallel. This notion would be supported by noting that in F_1 hybrids of $K \times A$ (Crozier and Pincus, 1929-30, *a*), where θ -area is intermediate between those for the parental stocks, total variation and percentage controllable (α 15° to 70°) are also intermediate, but the variability number is *below* that for A . Other data on different lines, however, seriously disturb these apparent correlations. There is no necessary correspondence among the indices of variation and the "number of receptor units" when different lines are compared, although when dealing with individuals of a given stock such parallelism may well be expected. Since the method of estimating the "number of receptors" implicitly defines the number in terms of functional units, this situation is entirely reasonable. The parallelism of *proportionate relative variation* to areal measure of quantity of excitation units is closer if one ventures to extrapolate to the "ideal" threshold for excitation of the receptors of Group 1 which may be supposed to undergo excitation at threshold slope. This question is better examined with other lines of rats; it is closely connected with the relation of threshold $\sin \alpha$ to θ at the threshold.

IX

The remaining point to be examined is the possibility of homologous modification of the specific curves for different homozygous lines under similar experimental change of the conditions determining θ as a function of α . This should supply a test of the *kind* of analysis accorded the two curves. We have shown (1929-30, *b*) that when a mass of about 2 gm. is attached at the saddle of young rats of line K the curve connecting θ with $\log \sin \alpha$ is distorted in a way which can be understood if a good number of receptors of Group 2 are brought into action, owing to the presence of the attached mass, at a much lower inclination of substratum than otherwise suffices to do so. The question then is, concretely: Will the curve for A exhibit a similar distortion when rats of this line similarly carry an added load of about 2 gm.? The test of this point also supplies certain criteria for the variability

number and associated indices. It should be obvious that modification of this sort, if resulting in an entirely comparable disturbance of the analytical structure of the θ -curve, would add considerably to the force of the assumption that the structure is competent. A corollary which likewise must be examined is, that a similar mass carried in a different position on the body must distort the usual θ - α curve in a different way. Experiments with rats of line *A* give results which show that by these tests the differential arrays of "receptor groups" (Figs. 19, 22) afford a rational picture of the situation as regards geo-

TABLE VI

Data from tests for extent of upward orientation, with a mass of 2.12 gm. attached at saddle position ("posterior weight"); young rats of line *A*, 13 days after birth; $t^\circ = 21.3^\circ \pm 0.6^\circ$. For litter *A*₁₉, *N* = 4 (2 ♂♂, 2 ♀♀), weighing 16.0 to 18.5 gm., for *A*_{19a}, *N* = 2 (1 ♂, 1 ♀), for *A*_{18f}, *N* = 3 (all ♀♀), weights 17.0 to 18.0 gm.; *n* = 20 throughout. See text.

Litter	α , degrees	θ , degrees
<i>A</i> ₁₉	20	64.14 \pm 1.10
	30	69.94 \pm 0.986
	35	69.13 \pm 0.936
	45	73.46 \pm 0.830
	55	78.44 \pm 0.616
	70	78.08 \pm 0.580
<i>A</i> _{19a}	25	66.37 \pm 1.16
<i>A</i> _{18f}	25	65.32 \pm 0.976

tropic excitation and orientation in both line *K* and *A*. The further extension of these analytical experiments with added loads will be considered at some length later on.

Tests were made with *A* litters, each rat carrying a brass weight attached with chicle on the back at the saddle position; the total mass added was 2.12 to 2.13 gm. (cf. Crozier and Pincus, 1929-30, *b*, Fig. 21). It is to be noted that the looseness of the skin makes it impossible, even with flat weights of sheet brass, to be sure of constancy in the mode of pull exerted by the added load. Even with this handicap, however, the results are unequivocal (Table VI; Fig. 19). The manner

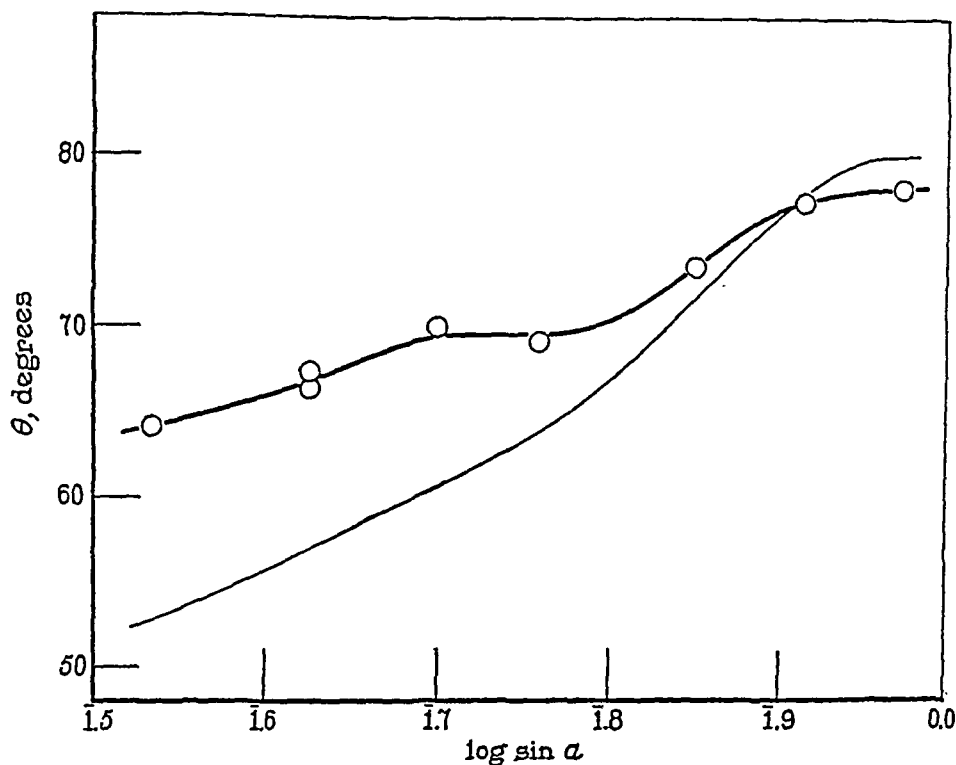


FIG. 19. Orientation-angle θ as function of slope of substratum, when a mass of 2.12 gm. is attached at saddle position of rats of line *A*. The curve for unloaded rats is transferred from Fig. 4.

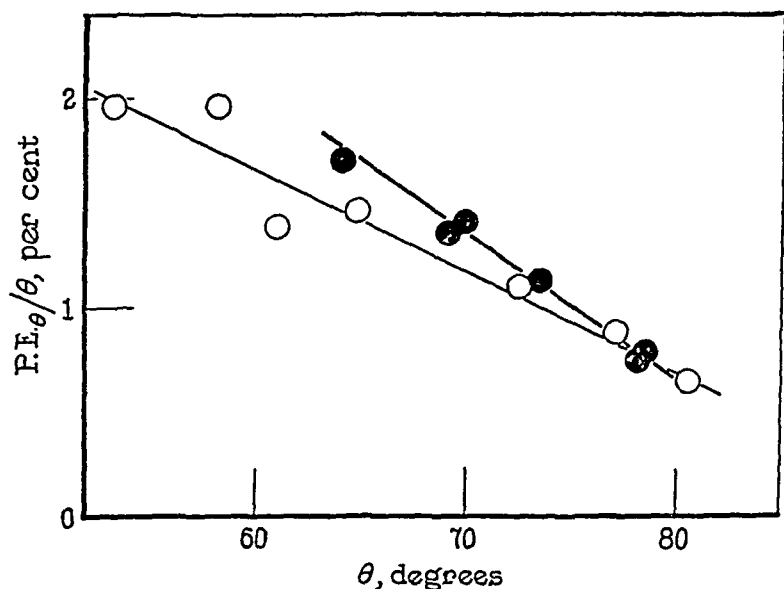


FIG. 20. Variability of orientation-angle in *A* rats with posterior load (A_{19} solid circlets); $V.N._\theta = 3.06$. For A_{18} , without load (open circlets), $V.N._\theta = 2.29$; and for A_{16} , without load, 2.58.

in which the θ -log $\sin \alpha$ curve is distorted by the additional mass carried is precisely that exhibited by the K curve with the same added mass carried in the same position. A low- θ segment is lifted, made a little less steep; a flat portion is apparent in the mid-region; the high- θ end of the curve cuts below that for orientation without the weight.

As with K , at given α the variation of θ (μ and N equivalent) is less with load than in its absence. At equal θ 's the variation is about the

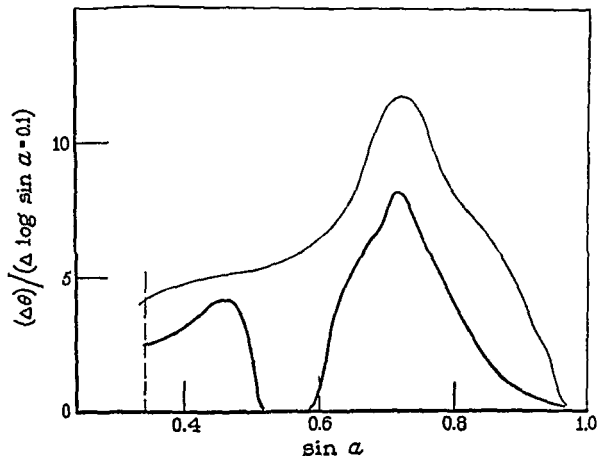


FIG. 21. $(\Delta\theta)/(\Delta \log \sin \alpha)$ vs. $\sin \alpha$ for A rats with posterior load. The corresponding graph for the unloaded rats is transferred from Fig. 17.

same with load and without; V.N. computed from $\Delta(P.E._\theta/\theta)/\Delta\theta$, with $\Delta\theta = 20^\circ$, is 3.06 (Fig. 20), without loads (see Fig. 7) it is 2.29 to 2.84. With line K the variability was less on this basis, and with A the proportion of modifiable variation is markedly reduced. A test of the relation of variation to θ at threshold α cannot be made with A as yet, since it is probable that the threshold slope has been reduced below $\alpha = 20^\circ$ by the posterior weight.

The differential curve, Fig. 21, is of the type found for K individuals

with the load similarly placed. The effect of the posterior weight is exerted through action upon each of the three groups of receptors, but particularly upon 1 and 2; the dissection of Group 2 from Group 1 is in this way made clear.

The area under the differential curve is 7.12 units, as compared with 10.3 units in the absence of load. The ratio $10.3/7.12 = 1.45$ cor-

TABLE VII

Two series of tests, rats of line *A*, 13 days after birth, 2.12 gm. on back at shoulder level ("anterior position"); A_{18} ($n = 20$, $N = 3$), A_{19} ($n = 20$, $N = 2$); temperature $20.5^\circ \pm 1.0^\circ$. (These individuals also tested for orientation with posterior weight, and for orientation without added load.) Inclination of surface = α , mean orientation angle = θ . See Fig. 22.

α , degrees	θ , degrees	
	A_{18} degrees	A_{19} degrees
20	51.25 \pm 1.18	49.50 \pm 1.91
25	55.22 \pm 1.24	54.19 \pm 1.31
30	61.13 \pm 1.07	
35		65.65 \pm 1.04
40	73.57 \pm 0.655	
45		74.70 \pm 1.14
50	78.21 \pm 0.758	
55		83.87 \pm 0.510
60	81.16 \pm 0.602	
70		83.23 \pm 0.663

responds closely to the ratio of the total proportionate variations in the two cases: $56/39.5 = 1.42$.

The same additional weight differently disposed could not produce the same effect upon the θ -curve, if our understanding of the case is adequate thus far. Tests were made with the same mass (2.13 gm.)

attached on the back at shoulder level. The data are given in Table VII, plotted in Fig. 22. In this figure the central lines for θ without added weights, and for θ with the weight posteriorly located, have been included. The differences are obvious.

When the added mass is carried anteriorly the conditions are mechanically altered in two respects: an added load is moved, and the

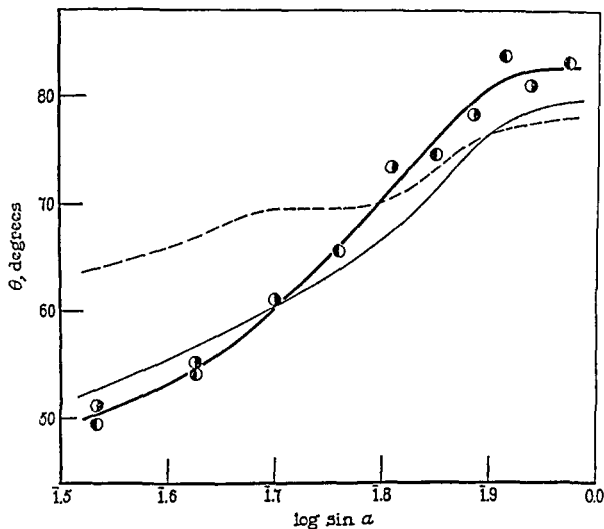


FIG. 22. Orientation-angle as related to inclination of surface, for *A* rats with 2.13 gm. attached to back at shoulder level. Curves for *A* rats without added load and with load 2.13 gm. located posteriorly are transferred from Figs. 4, 19.

center of gravity is shifted toward the head and upward. The latter change tends to endanger stability; regardless of the total mass moved, the rat must then turn to a higher θ , at given (high) α , to achieve sensory equivalence of tension excitation on the two sides of the body. This effect must be combined with the influence of a differently excited

array of receptor units. The situation is to this extent not unlike that in *Uca* (Kropp and Crozier, 1928-29). The net result of both effects is to produce a much more rapid general increase of θ with increasing α than in the absence of the anterior load. Since the posteriorly located weight moves the centroid caudally, it should not affect mechanical stability during progression to anything like the same extent. We need not expect, then, that the curves with pos-

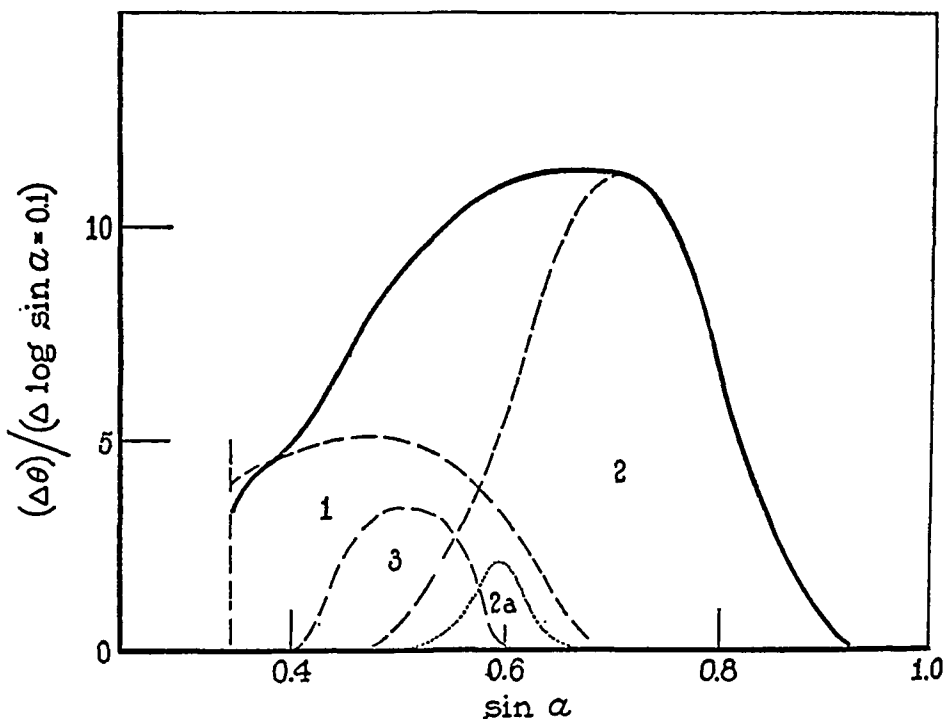


FIG. 23. $(\Delta\theta) / (\Delta \log \sin \alpha)$ vs. $\sin \alpha$, for *A* rats with added load at shoulder level. The curve is resolved into the three typical sub-areas, shifted from their positions in the absence of anterior load, plus area 2a; see text.

terior and with anterior weights will be in all respects comparable, since additional new types of tensions may be brought into play by the weight anteriorly placed. The unravelling of these effects requires, for one thing, experiments in which a mass of 1 gm. is carried at the saddle, and 1 gm. at shoulder level; but these need not concern us at the moment, since all we look for now is indication that diversely located weights act diversely upon our array of receptors, and par-

ticularly upon the three large groups distinguished in the antecedent analysis. Fig. 23 shows that the search is not unrewarded.

With the mass carried anteriorly, the variation of θ is not materially affected; the variability number, figured as $\Delta \frac{P.E.}{\theta} / \Delta \theta$, and corrected as before for n and N , is (Fig. 24) 2.36 to 2.84; this cannot be held really divergent from the corresponding index in the absence of loads, or even when the load is in the posterior position (*vide supra*). It is

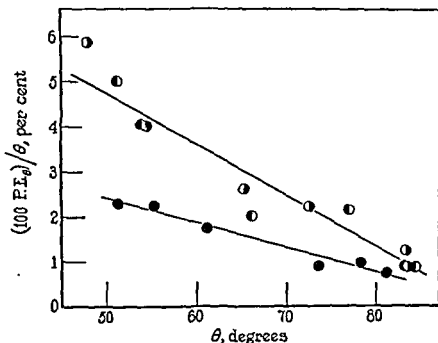


FIG. 24. $(\Delta P.E._\theta) / (\Delta \theta)$ vs. θ , for A rats with anterior load. For A_{18} , (solid circlets), $V.N._\theta = 2.84$. With A_{19} , the two individuals used differ slightly as to θ 's, and $V.N.$ must be computed separately; it is 2.3 from the line drawn. The proportionate variation (per cent of total modifiable) is 44 per cent in the first case, 75.7 per cent in the second.

to be remarked that the way in which the variation of performance keeps parallel in its behavior to θ is entirely consistent with the idea that θ is a measure of the excitation at orientation.

The differentiation of the θ -log $\sin \alpha$ curve for A rats with anterior weight results in Fig. 23. The resolution of this graph can be made as indicated in the figure. The area of sub-curve 2, measured after the analysis, is 6.20 units, identical with the area (6.18) obtained for 2 in the absence of weight (Fig. 17). The form of 2 is very slightly

modified, however; the distribution is flattened a bit and as a whole moved to a slightly lower $\sin \alpha$. The absence of group 3 from its usual position permits a test of the form of the curve for 2; it will be noticed that the right-hand margin of the symmetrical curve is simply the result of plotting $\Delta\theta/\Delta \log \sin \alpha$ against $\sin \alpha$; it is probably more than a coincidence that in spite of the chances of curve-fitting the areas of 2 should come out identical with the anterior weight and without. Group 1 area is again practically unchanged, its left-hand outline very slightly altered. Group 3 is brought into action at much lower slopes. The additional area $2a$, 0.36 units, is required to complete the construction of the differential curve as found. Noticing that θ_{max} (at $\alpha = 70^\circ$) is definitely greater with the anterior load, 83° as compared with 80° in the absence of load, it is natural to relate the increase to a greater number of receptor units involved. On this basis $83^\circ - 80^\circ = 3^\circ \equiv 0.36$ excitation units, and 1° of orientation angle $\equiv 0.12$ units of receptors. The total areas under the curves in Figs. 17 and 23 are respectively 10.3 and 11.0 units, almost exactly in proportion to the maximum θ 's attained, whence $1^\circ\theta \equiv 0.13$ units of area; sub-group $2a$ should thus correspond in area ($0.36 \pm$) to about 3 orientation units brought into action above $\alpha = 15^\circ$. In terms of the notion used for the curves with race *K* (Crozier and Pincus, 1929-30, *b*), the total areas under the differential curves (*i.e.*, the numbers of excitation units), ought to be proportional to the differences between θ_{max} and θ at threshold slope; inaccurate location of lower thresholds for excitation, and disturbances due to shifts of centroid (particularly with weight in the anterior position) interfere with this estimation.

The nature to be ascribed to area $2a$ in Fig. 23 is of course obscure. It may perhaps correspond to the effect of the altered position of the center of gravity in bringing into play a "new" set of tension receptors; or it might represent a functional dissection of the asymmetry of our "Group 1" which is suggested at its left-hand side. In either case, if we subtract its area from the total, leaving 10.7- units, the ratio of increase of θ above threshold response to area becomes 2.8, corresponding to 2.6 for the case without load, 2.25 with posterior load (assuming for the latter instance threshold $\alpha = 20^\circ$, which is probably a little too high; if lower, the ratio would agree more nearly with the two others).

The effect of the anteriorly-located weight clearly suggests that sense-organ Group 3 is located in the anterior legs. The possibility of more detailed localizations is fairly obvious.

SUMMARY

Extension of analysis of the functional basis of geotropic excitation and response in young rats has made it desirable to obtain, for additional genetically stabilized lines, further tests of the quantitative reproducibility of orientation data as secured from successive generations in these lines over a period of several years; and of the measures of variation of performance as these are related to the exciting vector and to the extent of orientation. Procedures are illustrated whereby the significance of measurements can be checked automatically. It is shown that differences apparent in the geotropic behavior of three inbred lines of *R. norvegicus* are quantitatively recoverable over a period of nine generations. The constant, characteristic features for each inbred line concern: the extent of upward orientation, absolutely and as a function of the inclination of the substratum; the threshold slope for orientation; the dispersion of mean orientation-angles as governed by the slope of substratum; the dependence of the dispersion of the relative variation of observed orientation-angles upon the intensity of excitation; and the proportion of the total variation of response which is modifiable as a function of the slope of surface.

It is also shown that when for two lines of rats the curves connecting orientation-angle with inclination of substratum differ in position and in details of form, the curves none-the-less undergo distortions of homologous type when rats of these lines creep geotropically with the same additional load in the form of a mass attached at a corresponding position on the back; and that shifting this mass to another position induces a quite different modification of the curve.

These effects are discussed in terms of the view that orientation during geotropic creeping is controlled by the adjustment of sensorially equivalent tension-excitation in the legs of the two sides of the body, and that the frequency distributions of thresholds for excitation within the several groups of receptor units concerned differ quantitatively among the inbred stocks, but are statistically constant within each line.

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ANALYSIS OF THE GEOTROPIC ORIENTATION OF YOUNG RATS. IV

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I

Observations utilized in providing material for the interpretation of orientation during the upward geotropic progression of young rats have been secured from three inbred lines of *R. norvegicus*,¹ hybrid descendents from two of these,² and one line of *R. rattus*.¹ It has been desirable to obtain comparable data upon certain other lines. Experiments were undertaken with individuals of a line labelled *P*, started some years ago by inbreeding from the original stock which had also been the source of lines *A* and *B* of our previous accounts.³ It was expected that certain resemblances, and possibly some differences, would characterize lines *A*, *B*, and *P* and serve to differentiate them as a group from rats of line *K*.² Evidence of this sort would amplify the proof that differences and similarities in the properties of geotropic behavior are correlated with hereditary differences and similarities among the tested individuals.

Race *P* was derived from the same original source as our *B* and *A*, namely, a miscellaneous group of rats arising from certain studies of linkage in rats made at the Bussey Institution of Harvard University and subsequently bred brother to sister. They differ from lines *B* and *A* in being pink-eyed, agouti, hooded. The former are (except for line *B'*) red-eyed, non-agouti, hooded.

Line *K* of our previous accounts^{1,2} originated from a quite different source. It is totally unrelated to lines *A*, *B*, and *P*, deriving from a pair of rats obtained from Miss Helen D. King in 1924.

¹ Crozier and Pincus, 1926-27, *a*, *b*, *c*, *d*. 1927-28; Pincus, 1926-27.

² Crozier and Pincus, 1929-30, *a*, *b*.

³ Crozier and Pincus, 1931-32 (III).

We accordingly look for rather definite similarities in the results to be obtained with A , B , and P individuals, which will collectively differentiate them from K rats. We may further expect, on the basis of earlier findings demonstrating genetic stability of geotropic performance within inbred lines,³ that these resemblances will not only include the relationship of orientation-angle θ^1 to angle of slope of surface (α), but will extend to the *variability* of the orientation-angle. These expectations are in fact met by the measurements. The findings are also of interest because they can be interpreted to show that certain of the effects distinguished in cross-bred individuals (*e.g.*, $K \times A$; Crozier and Pincus, 1929-30) may be simulated in a "pure line" of rats. The result of this, in line P , is a disturbance of the approximate rectilinearity of the $\cos \theta$ *vs.* $\sin \alpha$ plot.¹ By chance the races with which we first worked^{1,2} did not exhibit this peculiarity, $\Delta \cos \theta / \Delta \sin \alpha$ being very nearly constant; though in hybrids of A and K , and in the back-cross offspring of that experiment, it is quite apparent and has been interpreted.³ The condition revealed in P might of course be due perhaps, or in part, to a different disposition of the relative masses of the parts of the body, as compared, let us say, with race A ; there are other possibilities, but the explanation apparently turns upon specific differences in the "number of receptors" in one of the three main groups distinguished in the analysis of the θ -log $\sin \alpha$ curve.^{2,3}

II

Measurements with three litters of line P are summarized in Table I, and the respective mean orientation-angles are plotted in Fig. 1.⁴

⁴ It is noteworthy here, as with the data from tests with race A ,³ that in the θ *vs.* log $\sin \alpha$ plot (Fig. 1) the mean θ 's tend very definitely to exhibit a strictly limited scatter; marginal lines fairly including the plotted points are very closely constant in their separation in the abscissa direction. This means that the ratio of the lowest $\sin \alpha$ required to evoke a given mean θ to the highest $\sin \alpha$ ordinarily evoking that θ -response, is constant. Consequently, to obtain a *definite* increase of mean θ , one must increase mean $\sin \alpha$ giving that θ by a constant fraction; the total range of $\sin \alpha$'s evoking a given average θ is, for the P line, = about 10 *per cent* of the lowest $\sin \alpha$ giving mean θ of that magnitude for a sample of the sizes (n) used. It is to be noted, however, that the "definite increase of θ " thus evoked is not constant, but is a function of $\sin \alpha$.

Litter I, five individuals (3 ♂, 2 ♀) was of the fourteenth brother × sister inbred generation (P_{14}); litter II, four individuals (2 ♂, 2 ♀), resulted from a mating of a P_{14} female with a male derived from litter mates of P_{13} ; litter III, three individuals (2 ♂, 1 ♀), was produced in another mating similar to that giving II. All were used 13 days after birth. In each case twenty observations of θ were secured with each

TABLE I

Orientation-angles (θ) at various inclinations of surface (α), young rats of race P . Three litters: No. I, five individuals; No. II, four individuals; No. III, three individuals; $n = 20$ with each individual at each slope.

α degrees	θ , degrees		
	P_{14} I	P_{14} II	P_{14} III
15	47.9 \pm 1.18		49.5 \pm 1.24
20	55.0 \pm 1.17	54.9 \pm 1.15	
25	57.8 \pm 0.990	59.4 \pm 1.03	
30	61.7 \pm 0.895		63.2 \pm 0.873
35	68.4 \pm 0.795	66.2 \pm 0.888	68.1 \pm 0.802
40		68.0 \pm 1.00	71.7 \pm 0.682
45	73.1 \pm 0.746	74.1 \pm 0.975	
55	77.7 \pm 0.494	77.4 \pm 0.714	
62.5		78.1 \pm 0.643	
70	80.2 \pm 0.586		79.9 \pm 0.524

individual at each slope (α). The differing number of individuals in the three instances facilitated certain checks upon the "variability number" subsequently computed. For Series I, II, III the respective temperatures were: 20.4° \pm 0.3°; 22.5° \pm 0.5°; 22.1° \pm 0.4°, the limits here indicating the extreme ranges. The methods of recording oriented paths are given in a preceding paper (Crozier and Pincus,

1931-32). The three series are satisfactorily concordant; one of them, as with line *B*,³ tends to be *slightly* higher throughout.

The form of the curve connecting orientation-angle θ with $\log \sin \alpha$ is that previously found with other lines^{1,2,3}. It resembles those for lines *A* and *B*, and with them differs distinctly from that for line *K*. The *P* curve most closely approaches that for the *A* rats, but θ is

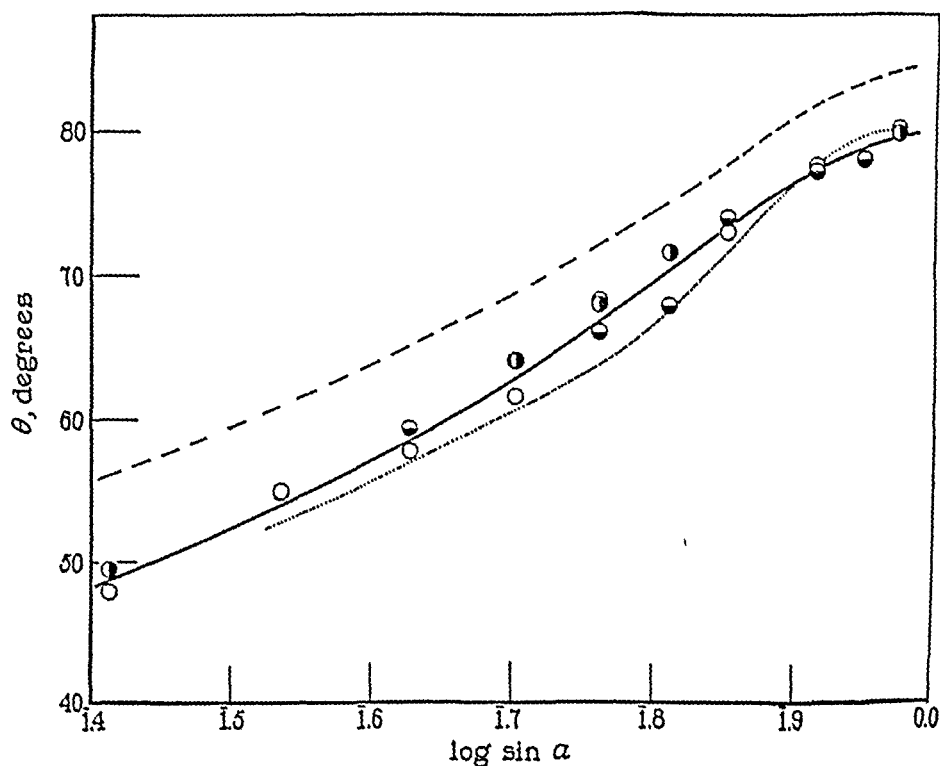


FIG. 1. Mean values of orientation-angle θ obtained with three litters of line *P*. Data in Table I. Curves obtained with lines *A* and *B* are transferred from graphs previously published (Crozier and Pincus, 1931-32).

consistently higher than with *A* except above $\alpha = 55^\circ$. Whereas with *A* no consistent orientation has been obtainable at $\alpha = 15^\circ$, it is regularly secured at that inclination of surface with *P* and with *B*. *A* rats require a higher threshold slope (20°) for orientation, and show a lower orientation-angle at that inclination of surface, than do *B* or *P*. These two facts cannot be directly related, however, because *K* rats, with still lower θ 's at low slopes, respond clearly and definitely at $\alpha =$

15°. When lines *A* and *B* are crossed, as described in a later paper, the F_1 individuals show the threshold of slope and the threshold θ of the *B* parents. Although the curve for *P* is superficially closer to that for *A* than for *B*, it will be shown in the analysis of the graph that its fundamental similarities are with the *B* curve rather than with the *A*. In view of the fact that on the basis of mere tabulation of the data and their standard deviations it might be suggested that *A* and *P* curves are not really distinct, cases of this type again illustrate the necessity of considering response or performance as a function of measured variables (*cf.* Crozier and Pincus, 1929-30, *a*). Testing the response of *A* and *P* individuals by estimations of θ at one value of α would leave one no choice but to decide them so similar as to be

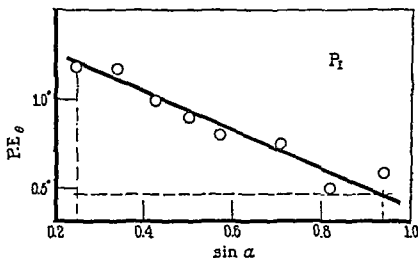


FIG. 2. The σ of mean θ declines rectilinearly as $\sin \alpha$ is increased. Series I (*cf.* Table I, and Fig. 3).

practically indistinguishable, although this error would result from a rather different cause than that which could easily lead to similar confusion if *K* rats were incompletely compared with either *A* or *P*.

As in the earlier experiments,^{1,2,3} $P.E._\theta$ declines rectilinearly with $\sin \alpha$ (Crozier and Pincus, 1931-32), and $P.E._\theta/\theta$ with $\log \sin \alpha$ — the θ vs. $\log \sin \alpha$ plot being again nearly enough rectilinear for the purpose of this test (which otherwise⁴ would be made by plotting $P.E._\theta/\theta$ against θ). [*Cf.* Figs. 2, 3; 4, 5.] The “variability numbers” computed from the slopes of the lines in Figs. 4, 5 are collected in Table II. This quantity is obtained (Crozier and Pincus, 1929-30; 1931-32; Crozier, 1929) as $\Delta (100 P.E._\theta/\theta)/\Delta (\log \sin \alpha)$, multiplied

by $\sqrt{n/N}$, where n = number of observations upon each individual, N = number of individuals in the group (litter); it signifies the mean

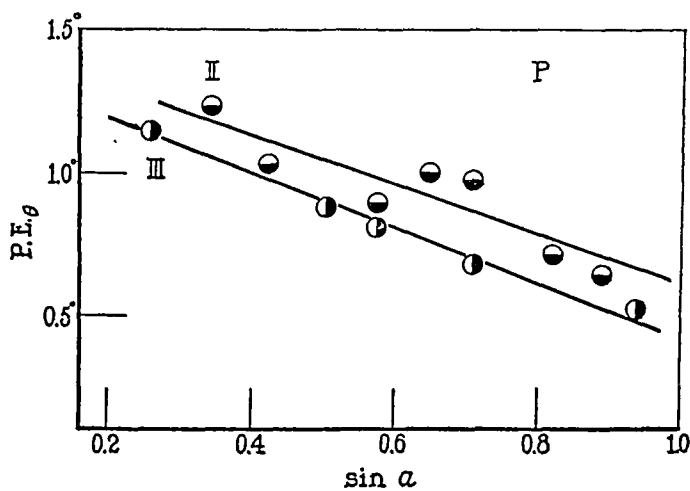


FIG. 3. $P.E._\theta$ vs. $\sin \alpha$ for Series II, III (cf. Fig. 2; data in Table I)

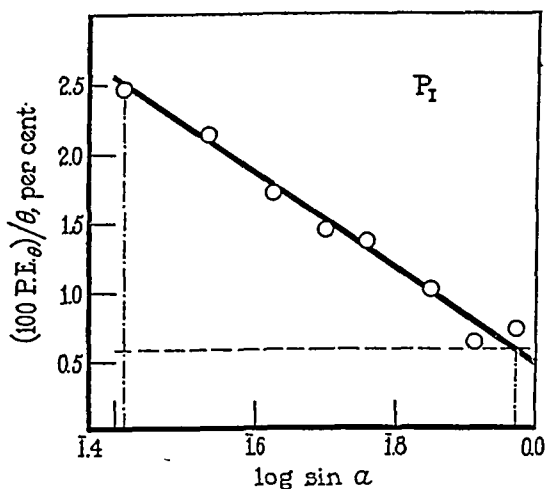


FIG. 4. The relative variability of the geotropic orientation ($P.E._\theta/\theta$) declines rectilinearly as $\log \sin \alpha$ is increased. Series I (cf. Fig. 5).

percentage change of the mean root mean square deviation from the general mean θ , per individual, per unit change of the independent variable ($\log \sin \alpha$). The three series in Table I agree remarkably

well, and permit comparisons with the corresponding indices already computed for the other races (Table II). Of the three series, I is the most reliable, partly because observations were taken at a larger number of slopes (α). In Series III one individual gave particularly low σ_θ 's; this rat, ♀, was much below the weight of the others, al-

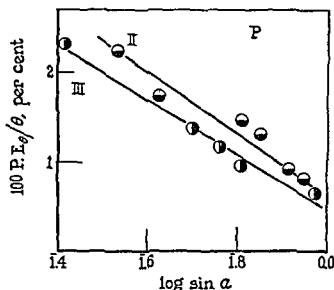


FIG. 5. $P.E._\theta/\theta$ as function of $\log \sin \alpha$, Series II, III (cf. Fig. 4)

TABLE II

Variability numbers for geotropic response in four races of *R. norvegicus*. Mean values for K , A , B , taken from a previous paper.³

Line	V.N. from $\Delta(P.E._\theta/\theta)/(\Delta \log \sin \alpha = 0.4)$
K	5.78
A	2.84
B	3.32
P	2.86

though its mean θ 's showed no differences from those of the other individuals; it weighed 14.2 gm., its litter mates 20 gm. The rats of litters I and II weighed 15–16 gm. Careful inspection of individual records has demonstrated for the P race, as with earlier data for A , K , B ,^{2,3} that there are no correlations of geotropic responsiveness (θ)

with individual weight, or with sex. In Series II the observations at two slopes ($\alpha = 40^\circ, 45^\circ$) were taken in the evening, when variability of performance is greater, but the distortion is not serious (Fig. 3, 5) although definitely detectable. (We shall later consider this aspect of the measurement of variability in greater detail.) The variability numbers 2.76, 3.02, 2.84 are in unusually good agreement, and the weighted mean is plainly of the same order of magnitude as in lines *A* and *B* (Table II)—*P* and *A* are indeed not really distinguishable in this respect. The correspondence of this fact with the known common derivation of the three lines is consistent with the different magnitude for line *K*, and with the behavior of the variability number in

TABLE III

The percentage of the total observed relative variation of θ which is modifiable according to the magnitude of α .

Line	Proportionate relative variation
	<i>per cent</i>
<i>K</i>	85 ($\alpha = 15-70^\circ$)
<i>A</i>	56 ($\alpha = 20-70^\circ$)
<i>B</i>	74 ($\alpha = 15-70^\circ$)
<i>P</i>	59 ($\alpha = 15-70^\circ$)

breeding tests.^{2,3} V.N. cannot be very directly connected, it is clear, with the threshold for response, and attempts to characterize the relative geotropic sensitivities of the several lines on these bases would be in conflict.⁵

⁵ The *variability* of θ can be estimated in a slightly different way, by considering the data for each individual separately. Although this has been done for all our measurements, we prefer to keep together all the observations on each litter (with inbred lines). V.N. computed on the basis of mean P.E. _{θ_1} for a group, where θ_1 is the individual mean θ at given α , n being constant, turns out a little higher or a little lower than the values given, though the general average is the same. The chief reason for this is that in addition to slight differences in the slope factor $\Delta \text{P.E.}_\theta / \Delta \log \sin \alpha$, independent differences also occur in the ordinate intercept. In the *P* series the *individual* V.N.'s range from 2.0 to 3.2.

III

The *proportionate relative variation* of orientation (Crozier and Pincus, 1931-32) is obtained by considering the percentage of the total area under graphs such as those in Figs. 2, 3, corrected for n and for N , which is modifiable according to the slope α . In the experiments with line P the result is given in Table III. The percentage of the total relative variation of the orientation angles which is governable by $\sin \alpha$ is 59.4 *per cent*, the weighted average of 62.5 (Series I); 58.9 (Series II — extrapolating to $\alpha = 15^\circ$), 59 (Series III). This com-

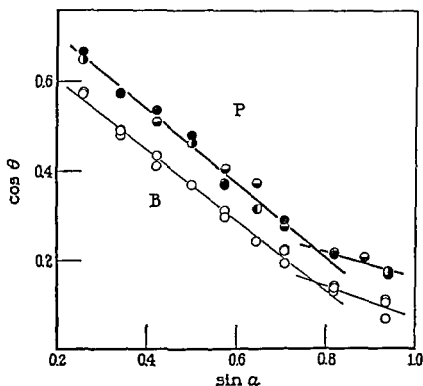


FIG. 6. $\cos \theta$ vs. $\sin \alpha$ for line P , three series

pares well, at first sight, with the value previously obtained with A rats (*cf.* Table III), but for A the range of α is only $20-70^\circ$. Comparing B and P , the two having the same threshold slope, the total relative variation is greater with P , while the proportion modifiable is definitely less.

IV

For the P race the graph of $\cos \theta$ vs. $\sin \alpha$ is not simple (Fig. 6). It corresponds to that for B rats,³ except that the θ 's are of course lower. The general form of the plot is somewhat like that for those segregates

produced in the back cross $F_{1(A \times B)} \times A$ in which receptor group 1 is small, group 2 large (Crozier and Pincus, 1929-30, 3, p. 101). The change of slope indicated in Fig. 6 can be demonstrated real, and not merely statistically significant, by the behavior of $P.E._{cos \theta}$ or of $P.E._g/3$, *vs.* $\sin \alpha$. In this respect it is analogous to the discontinuity in the corresponding curves established for guinea pigs (Upton, 1929-30; Crozier, 1929), although its nature is different. The identification of a discontinuity by such means is proof of its inherence in the data (*cf.* also: Hoagland and Crozier, 1931-32).

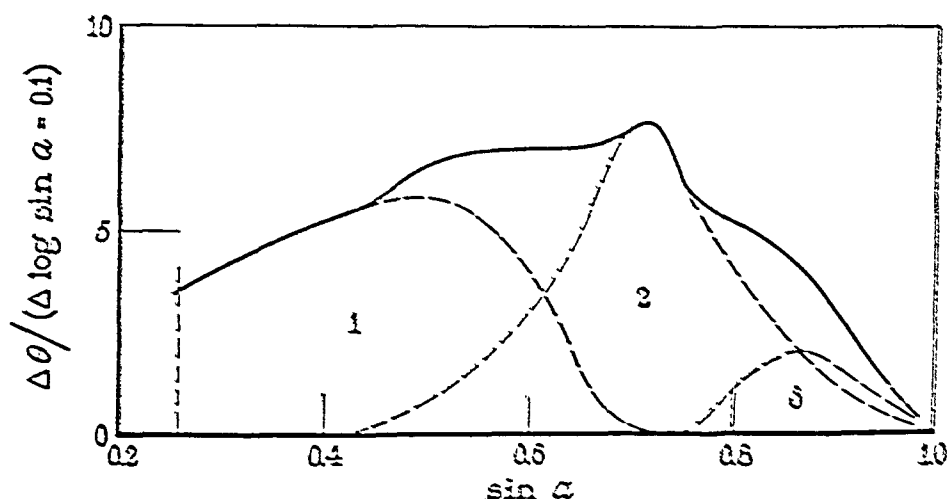


FIG. 7. $(\Delta \theta)/(\Delta \log \sin \alpha = 0.1)$ for line *P*, showing its resolution into three sub-regions analogous to those recognized with lines *A*, *A*, and *B*.^{1,2,3}

We have utilized the relationship between $\cos \theta$ and $\sin \alpha$ largely as a matter of convenience,^{1,2,3} because for races *A*, *A*, *B*, and for our stock of mice (Crozier and Oxnard, 1926-27) and of *Rattus rattus* (Crozier and Pincus, 1926-27), it gives a sufficiently rectilinear plot. The deviations from a simple straight line, in such representations, are none the less definite and real (Pincus and Crozier, 1929). With cross-bred individuals this is very clear (Crozier and Pincus, 1930-31). For race *B* we were inclined to ignore the indications of discontinuity at about $\alpha = 50^\circ$ (3), pending further data; but more recent measurements with *B* rats show it to be definite, and the behavior of *P* discloses an exactly similar state of affairs. If we were to take the $\cos \theta$ *vs.* $\sin \alpha$ plots as a basis for analysis, we could point to the pres-

ence in "pure line" *P* of components of reactivity somewhat resembling those brought into single individuals by breeding experiments with lines *K* and *A*.²

v

It was shown previously^{2,3} that by taking into account the fact that the act of stepping provides the excitation which controls the geotropic orientation during progression, the slopes $\Delta\theta/\Delta \log \sin \alpha$ of curves such as that given in Fig. 1 can be employed to obtain a picture of the distribution of effective thresholds for excitation in the array of receptors concerned. For race *P* this is given in Fig. 7. As with the lines of rats earlier used, the curve is obviously composite, and is resolvable into three distinct parts or regions. When we compare the graph with those for races *A* and *B* it is clear that the affinities of *P* are with the *B* line rather than with *A*. For *B* it was pointed out³ that the three "groups of sense organs" contributing to the composite distribution are "small" in *B* as contrasted with those for race *K* and more flattened than in *A*, but that 1 (see Fig. 7) was larger than in *A*, 2 and 3 smaller. The analysis in Fig. 7 indicates that a similar resolution of the curve is possible for *P*, which is much more like the result obtained with *B* than the findings for *A*. The areas under the three sub-curves are, in planimeter units on the same scales:

Group	1	2	3	Total
<i>A</i>	3.10	6.18	1.12	10.4
<i>B</i>	4.46	4.00	0.65	9.11
<i>P</i>	4.68	4.12	0.71	9.51

The form of the sub-curves is also slightly different in the three cases. The maxima occur at about the same values of $\sin \alpha$, that for *P* 2 a little below that for *B* 2.

This material enables us to interpret tentatively the meaning of differences of threshold slope of substratum for orientation, and also of the relation of variation of θ to total number of receptor units.

We may suppose that to be effective in determining orientation the

total excitation must exceed a certain value. In terms of the excitation-units presumed to be characterized by the frequency distributions in Fig. 7, we can as an approximation suppose that this means that a certain number of excitation-units must be brought into play. The fact that *central* thresholds and "resistances" perhaps vary from line to line of rats is probably to some extent taken care of by the nature of the units in which the elements of excitation ("receptor units") are defined (Crozier and Pincus, 1929-30, *b*). We might then look for threshold α to be that inclination at which $\sin \alpha$ is adequate to excite a certain number of receptor units. This number corresponds pictorially to the missing left-hand margin of the differential curves. The total excitation at threshold slope is, in our terms, measured by threshold θ . The magnitude of $\sin \alpha$ able to produce this intensity of excitation will depend on the form of the frequency distribution of area *I*. For *K* rats this "group of receptors" is large, its outline is steep, and θ at threshold slope is relatively low. On this basis we expect threshold $\sin \alpha$ to be relatively low for the *K* line; the threshold inclination is at about $\alpha = 15^\circ$. Curve *I* for *B* and for *P* definitely encloses a larger area than in the case of *A*, and we look for a higher threshold slope in *A*, which was found; the form of Curve *I* in *P* (as deduced from the analysis) is such that it is steeper, less spread than in *B*; we expect then with *P* a lower θ at $\alpha = 15^\circ$ than with *B*, which again accords with the findings. The attempt to deal with these points in greater detail is of course hindered by the experimental difficulty of locating threshold slopes precisely, and of attempting the reconstruction of the missing portion of Curve *I*; but even in comparing the results with lines *A* and *B* it can be predicted, on the basis of the forms of Curve *I* in the two cases, that if (as found with *A* and *K*) the respective group *I*'s are inherited alternatively in crosses involving *A* and *B*, that the rats possessing a "B" group *I* will also orient at $\alpha = 15^\circ$, those with "A" group *I* not until about $\alpha = 20^\circ$ is reached.

The question of the nature of the indices of variation of θ can be approached in a similar way. The areas under the differential curves (Fig. 7) indicate the numbers of effective receptor units above the threshold for response, in terms of the analysis we have employed. A given departure from mean θ is less likely to occur if the functional receptor units are more numerous. Consequently $\Delta \text{P.E.} \theta / \Delta \sin \alpha$

(cf. Figs. 2, 3) should be greater with larger total area under the differential curve, since the bringing in of relatively more receptor units by a given increase of $\sin \alpha$ should cause $P.E._\theta$ to decrease more rapidly. For A, B, P , $\Delta P.E._\theta / \Delta \sin \alpha$ is respectively (weighted means) 0.93, 1.11, 0.89 (calculated with $\Delta \sin \alpha = 0.4$); for K , with the area in same units = 17.4, $\Delta P.E._\theta / \Delta \sin \alpha = 1.6 \pm$ or a little larger. The correlation demanded is thus met about as precisely as could be expected. The correspondence is perhaps the more convincing since the actual orientation-angles are so much higher in B than A or P , while the differential curves are so nearly alike in B and P . In a similar way we might expect the proportionate relative variation to be greater in K than for B or P , which is clearly the fact; whereas with A the higher threshold α prevents direct comparison. The mean *total* relative variations with K, B , and P (corrected for N and n) are respectively 2.8, 1.5, and 1.9 units; that for A falls into place in the series if its orientation-threshold is extrapolated to $\alpha = 15^\circ$. The net result is to indicate that "geotropic sensitivity" is about equal in lines A and P , higher in B , still higher in K . It is to be noticed that this order is in no simple way correlated with the threshold slope (α) for response, nor is it reflected in the magnitude of the response at threshold—criteria often employed in the comparative evaluation of reactivities. It is obviously related, however, to the maximal response obtainable under comparable conditions (*i.e.*, without added loads). All of the relations indicated are rationally accounted for in terms of the frequency distributions of thresholds for activation of receptor units, and the indices of variation as dependent upon the total number of these units. The examination of this matter will be considered in a later paper.

SUMMARY

The geotropic orientation of young rats of a closely inbred line P , separated 6 years ago from a stock which gave rise also to the closely inbred lines A and B previously studied, has been measured. The curve connecting orientation-angle with inclination of substratum is in a general way similar to those for A, B , and with them differs markedly from that for race K of totally different ancestry. The variability of the response exhibits similar affinities. Although the

orientation-angles are rather close to those obtained with *A*, the threshold slope for *P* is that for *B*; analysis of the curve discloses how this fact is related to the character of the presumptive distributions of thresholds for the respective arrays of tension-receptor units. It is pointed out that these considerations prevent loose comparisons of "geotropic sensitivity" in different lines of rats, but make it plain why comparisons in terms of thresholds for response or of magnitude of response at the same threshold are ineffective. The estimated "number of excitation units," however, is found to correspond to the variability of orientation, and to the manner and extent whereby the dispersions of the measured orientation-angles are governed by the magnitude of the gravitational vector.

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A CHEMICAL EXPLANATION OF THE VARIABILITY OF THE GROWTH RATE

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The growth properties of a cell are essentially concentrated in the chromosomes. These are believed to consist of subunits, the genes, and each of these genes seems to be the carrier of a special property of the organism. Each gene must therefore be chemically different from all other genes of the same cell, because each brings about a different chemical (or physical) reaction. Few organisms are known which have duplicate or triplicate chromosome complexes. Multiplication of cells seems to be brought about primarily by the reactions of these genes which may be considered growth catalysts.

Division of a cell is preceded by a division of all chromosomes which means a division of all genes. This doubling of the genes is the final step of an elaborate synthetic process, but it is the only step which we can observe.

Since all genes in one cell are chemically different and since they do not appear to react with one another, it is probable that the doubling of each gene in a cell is independent of the doubling process of the other genes, and that we may consider the rate of doubling of one gene separately.

If we have a large number of uniform cells, containing the same number of molecules in the same arrangement, it may be that the same gene in all these cells will double at the same moment. With this assumption, all identical cells should multiply at the same moment. It seemed more probable to the author, however, that the doubling of a gene, being a chemical process, would follow chemical laws. Just as molecules in a solution do not all react at the same time, but follow a definite order, so the corresponding genes in a number of identical

cells may not double at the same moment, but follow a definite order. The simplest assumption, in analogy to the mass law, is that the rate of doubling is proportional to the number which have not yet doubled.

With this assumption, identical cells would not all double at the same time; some would divide faster than others, but this difference would be a matter of chance only, and would not be inheritable.

It is possible to compute the order in which cells would multiply if the latter assumption were true. Let us assume for the start a theoretical organism with only one gene. Let a be the number of identical cells, and let m be the fraction of genes doubling per unit time. The number of cells in this case is the same as the number of genes. We then find:

	Cells with gene unchanged	Cell with gene doubled
At the start.....	a	0
After 1 time unit.....	$a(1 - m)$	$a \cdot m$
" 2 " "	$a(1 - m)(1 - m)$ $= a(1 - m)^2$	$am + am(1 - m)$ $= am(1 + 1 - m)$
" 3 " "	$a(1 - m)^3$	$am[1 + (1 - m) + (1 - m)^2]$
" t " "	$a(1 - m)^t$	$am[1 + (1 - m) + (1 - m)^2 + \dots + (1 - m)^{t-1}]$ $= a[1 - (1 - m)^t]$

This deduction implies something analogous to a monomolecular reaction because it assumes that m is independent of the ratio gene: food, or that the concentration of food within the cell is not changed by the doubling of the gene. For well nourished cells, this assumption may be correct. The growth rate of bacteria is known to be independent of the food concentration above a fairly low limit.

If *two* genes must double before cell division can occur (the two genes reacting independently of each other), they would offset each other's chances for cell division to a certain extent, since one gene might react very early in a certain cell and the other very late. This would be an extreme case; the probability of the event that both molecules have reacted can be computed. If the probability that the first gene has doubled in a given time is P_1 and the probability that the other has doubled, is P_2 , then the probability that both genes in the

same cell have doubled is $P = P_1 \cdot P_2$. The probability that the first gene has doubled after the time t was found to be

$$\begin{aligned} P_1 &\approx 1 - (1 - m)^t \\ &= 1 - q^t \end{aligned}$$

if we substitute the continuously reappearing term $1 - m = q$; q is the proportion of not-reacting genes per unit time.

The probability that the other gene (doubling at the rate of n genes per unit time) has reacted, is

$$\begin{aligned} P_2 &\approx 1 - (1 - n)^t \\ &= 1 - q'^t \end{aligned}$$

The probability that both genes have reacted in the same cell is $P = P_1 \times P_2$. This formula can be simplified considerably by assuming that though these two genes multiply independently, they multiply at the same rate which means $m = n$, or $q = q'$. The probability that both genes have reacted in the same cell is then

$$P = (1 - q^t)^2$$

For any number of genes per cell, g , we would get the probability that all genes have reacted in the same cell, as

$$P = (1 - q^t)^g$$

This formula includes the assumption that all genes multiply at the same rate.

The computation of theoretical cases of this kind is easy. P , *i.e.* the probability that all genes are doubled, or that a cell can multiply, if plotted as a function of time, is a sigmoid curve. From this we can compute the probability that a cell will double at a certain time. This computation has been carried through for several different rates of doubling, and for different numbers of genes. In Tables I and II are given the percentages of cells which would multiply at successive time units; they represent frequencies or probabilities.

Fig. 1 (for $q = 0.5$) shows how this frequency is affected by the number of genes. If the first cases, with very few genes, are disregarded, the curves differ mainly in their relative position, in their distance from the zero point, *i.e.*, in the time elapsing before the first cell division occurs; this time increases with the number of genes. There is little

difference in the general shape of the curves. Fig. 2 shows the curve for eight genes placed over that for 128 genes, and the difference is very slight.

TABLE I
Percentage of Cells Doubling per Unit Time

	No. of genes per cell						
	1	2	5	10	50	100	500
When 90 per cent of all genes of each kind double per unit time ($q = 0.1$)							
During 1st time unit.....	90.00	81.00	59.05	34.87	0.52	0	0
" 2nd " "	9.00	7.01	36.05	55.56	59.98	36.60	0.64
" 3rd " "	0.90	1.79	4.40	8.57	34.62	52.78	59.96
" 4th " "	0.09	0.18	0.45	0.90	4.38	9.62	34.57
" 5th " "	0.01	0.02	0.05	0.09	0.45	0.90	4.33
When 80 per cent of all genes of each kind double per unit time ($q = 0.2$)							
During 1st time unit.....	80.00	64.00	32.77	10.74	0	0	0
" 2nd " "	16.00	28.16	48.77	55.75	13.00	1.69	0
" 3rd " "	3.20	6.24	14.52	25.79	53.93	43.11	1.80
" 4th " "	0.64	1.28	3.14	6.13	25.38	40.42	43.13
" 5th " "	0.13	0.28	0.64	1.27	6.12	11.66	40.42
" 6th " "	0.02	0.03	0.13	0.26	1.26	2.49	11.50
" 7th " "	0.01	0.01	0.02	0.05	0.25	0.50	2.52
When 70 per cent of all genes of each kind double per unit time ($q = 0.3$)							
During 1st time unit.....	70.00	49.00	16.81	2.82	0	0	0
" 2nd " "	21.00	33.81	45.60	36.12	0.90	0	0
" 3rd " "	6.30	11.87	24.79	37.12	24.51	6.48	0
" 4th " "	1.89	3.72	8.82	16.13	41.19	37.87	1.71
" 5th " "	0.56	1.12	2.77	5.41	21.94	34.05	27.91
" 6th " "	0.17	0.33	0.84	1.67	7.87	14.55	39.73
" 7th " "	0.05	0.11	0.26	0.51	2.50	4.87	20.20
" 8th " "	0.02	0.03	0.08	0.16	0.77	1.55	7.28
" 9th " "	0.01	0.01	0.02	0.04	0.22	0.43	2.15

The assumption that the doubling of genes obeys the mass law leads to the conclusion that uniform cells will not all multiply at the same moment, but show a definite variation of their growth rate. The range of this variation is practically unaffected by the number of genes. Relatively, the variability decreases with a larger number of

genes. From Table II it can be seen that in each case, about eight time units are required for the doubling of 98 per cent of all cells. But an organism with eight genes will start multiplying after the first time unit has elapsed, and the total time required for 98 per cent of all cells to multiply is nine time units, while an organism with 1000 genes will

TABLE II
Order of Growth
Computed for $q = 0.5$
Percentage of Cells Doubling per Unit Time

Time units	No. of genes per cell								
	1	2	4	8	16	32	64	128	1000
1	50.00	25.00	6.25	0.39	0	0	0	0	0
2	25.00	31.25	25.14	9.62	1 00	0.01	0	0	0
3	12.50	20.31	26.98	24.35	10.81	1.38	0.02	0	0
4	6.25	11.33	18.63	25.31	23.80	11.29	1.59	0.03	0
5	3.12	5.96	10.82	17.90	24.56	23.52	11.50	1.69	0
6	1.56	3.05	5.83	10.59	17.56	24.21	23.39	11.60	0
7	0.78	1.54	3.01	5.76	10.47	17.39	24.03	23.31	0.04
8	0.39	0.78	1 54	3.00	5.73	10 43	17.31	23.96	1.96
9	0 20	0 39	0 77	1 53	2.99	5.71	10.40	17.27	12.16
10	0.10	0.19	0 39	0 77	1 53	2.98	5.70	10.37	23.44
11	0.05	0.10	0 20	0 39	0 77	1.53	2.99	5.71	23.80
12	0 02	0 05	0 09	0 20	0 39	0 78	1.53	3.01	17.20
13	0.01	0 02	0 05	0 09	0 20	0 38	0 77	1.51	10.00
14	0.00	0 02	0 02	0 05	0 09	0 20	0 38	0 77	5.40
15*			0 02	0 02	0 05	0 09	0 20	0 38	2.98
16			0 01	0 02	0 02	0 05	0 09	0 20	1.50
17				0 01	0 02	0 02	0 05	0 10	0 77
18					0 01	0 02	0 02	0 05	0 38
19						0 01	0 02	0 02	0 20
20							0 01	0 02	0 10

* All data for more than fourteen time units have not really been computed, but are derived from the parallelism of the other curves.

show practically no multiplication during the first seven time units, and a total of fifteen time units is necessary for 98 per cent of all cells to multiply. With the first organism, variability spreads over $\frac{8}{9}$ = 89 per cent of the total time, with the second organism only over $\frac{9}{13}$ = 53 per cent.

Even with 1000 genes, the "variability" due to chemical laws is considerable, and should be quite conspicuous in all experiments on growth

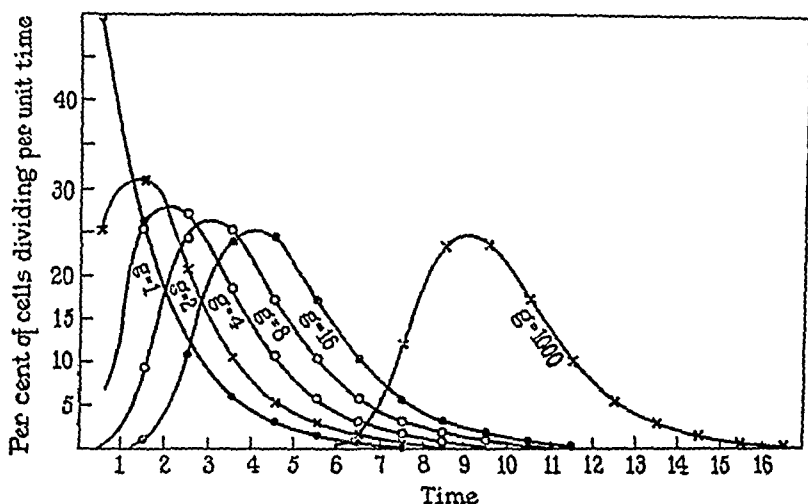


FIG. 1. Percentage of cells dividing in successive time units when the number of gene-type molecules per cell varies from 1 to 1000. (data computed for $q = 0.5$)

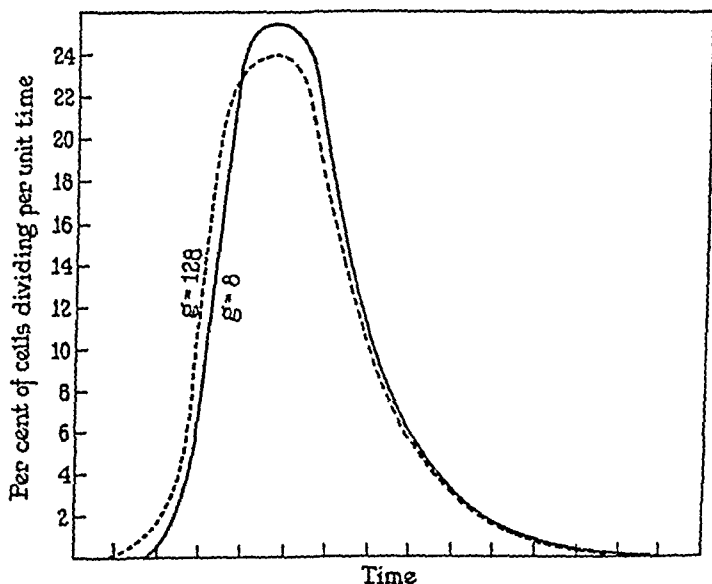


FIG. 2. Two curves for different numbers of genes from Fig. 1 drawn one over the other to show their similarity.

rates. With an organism possessing many genes, the growth rate should be less variable, relatively speaking, than with a simpler organ-

ism possessing only a few genes. This seems contrary to the author's "feeling." He would have reasoned that an organism possessing many genes, and therefore many properties, would show a wider range of variability of growth rate, being subject to more chances.

Another interesting fact is that the curve remains asymmetrical. Asymmetry is to be expected when the number of genes is very small, but the data in Tables I and II show a great constancy of the asymmetry even when the number of genes reaches 1000, and the same would be the case with a million genes.

EXPERIMENTAL EVIDENCE

In the introductory paragraphs, two possibilities for the doubling of genes in uniform cells were mentioned: either the same gene in all identical cells doubles at the same moment, or the doubling follows the mass law. The consequences of both assumptions have been discussed theoretically. It remains to compare both theories with the experimental evidence.

With bacteria, the customary method of measuring growth by plating is not applicable because we must obtain data on individual cells, while the plate count gives averages only. In a rapidly growing bacterial culture, we have all different stages of cell division simultaneously, and for the measurement of the order of growth, we must have all cells at the same starting point. Thus, only direct microscopic observation is likely to give us the desired data.

In one case only can the plating method be used, namely, in the germination of spores. We may consider all spores at the same stage of development. The order of spore germination has been measured by the plating method by Eijkman (1912-13), and he found the remarkable fact that the spores germinate in logarithmic order. There may be a very short period in which spores show no germination but as soon as they start, the rate of germination is proportional to the number of ungerminated spores. Fig. 3 shows the percentage of spores germinating per unit time.

The direct microscopic measurement of the generation times of a number of individuals is possible by using the agar hanging block (Orskov, 1922). K. A. Jensen (1928) gives a few data on the growth of *Bacterium coli*, but the number of individuals was too small and the

time intervals of 30 minutes too long to use these results for our purpose.

Mr. C. D. Kelly, together with the author (1931), has made a number of measurements of the growth rate of *Bacterium aerogenes* and *Bacillus cereus*. The data obtained with the latter organism cannot be applied here because spore-forming bacilli, at the period of fastest growth, sometimes do not form the cell walls separating the two new cells from one another, and no other indicator of the accomplished cell division was available. With *Bacterium aerogenes*, ten complete experiments were performed. These must be divided into four groups because the mode of the growth rate was not the same in all of them, and for our purpose, only such experiments can be summarized which

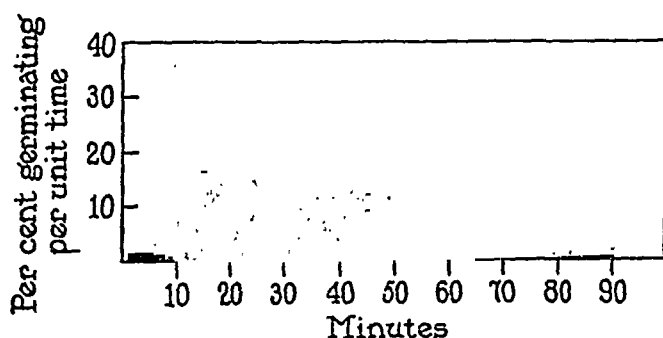


FIG. 3. Percentage of bacteria spores germinating in successive 10 minute periods.

show the same mode. Table III shows the three largest groups, with 733 fission times observed under the microscope. All three sets of data are skewed to the left.

These observations include usually four generations. If the variations observed in the growth rates are due to the chemical mass law, then they are a matter of chance, and the cells deriving from very slow and from very rapid fissions should have the same chances as all others. This is actually the case. No inherently slow or rapid growing strains were observed, and a rapid division was sometimes succeeded by a slow one, more commonly by an average one.

Table IV shows the fission times of the progeny of the 109 fastest growing individuals and of the 48 slowest growing individuals.

The same experiment was carried out with a yeast, *Saccharomyces*

ellipsoideus. The intervals were again 5 minutes. Except for the longer time required, the results are quite similar, as the two series in Table V show.

Here again, the frequency curve is distinctly skewed. In Series A, the six cells (2.7 per cent) dividing at the very rapid rate of 45 to 50

TABLE III

Frequency of Fissions of Bacillus aerogenes
(Number of Fissions Observed in Successive Time Intervals of 5 Minutes)

Time interval No...	2	3	4	5	6	7	8	9	10	11	12	13	14	Over 14	Total
Feb. 17.....					2	13	12	7	7	2	1				44
" 24.....			2	3	4	20	16	11	2	1		1			60
Mar. 3.....		1	3	12	20	38	19	18	8	4	1	1		1	126
" 10.....			6	10	16	26	18	9	3	1				4	93
Total	—	1	11	25	42	97	65	45	20	8	2	2	—	5	323
Percentage...	—	0.3	3.4	7.8	13.0	30	20.7	13.9	6.2	2.5	0.6	0.6	—	1.5	

Mar. 2.....	1		3	19	22	21	10	5	2	1					84
" 6.....			4	12	30	19	15	1		2	1				84
Total.....	1	—	7	31	52	40	25	6	2	3	1	—	—	—	168
Percentage...	0.6	—	4.1	18.5	31.0	23.8	14.9	3.6	1.2	1.8	0.6	—	—	—	

Mar. 17.....		6	23	43	30	9		1							112
Nov. 12.....			13	60	24	3									100
" 14.....			5	13	4	5	2	1							30
Total.....	—	6	41	116	58	17	2	2	—	—	—	—	—	—	242
Percentage...	—	2.4	17.0	48.0	24.0	7.0	0.8	0.8	—	—	—	—	—	—	—

minutes (10th interval) are the progeny of one cell and the two cells (0.9 per cent) dividing between 60 and 65 minutes (13th interval) are sister cells to these. Here was a distinctly inherent faster growth rate noticeable which is not due to chemical variation; it is the only example observed in this investigation.

No data could be found which give the actual rate of multiplication of protozoa, though probably some such experiments obtained under uniform conditions are available somewhere. The same pertains to data on algae.

The experimental evidence with bacteria and yeasts is in accordance with the assumption that the doubling of genes follows a chemical law. The variability curves are skewed to the left, as this assumption demands. The variation is relatively smaller with yeast than with

TABLE IV

Frequency of Fissions of the Most Rapidly and the Most Slowly Reproducing Individuals of Bacterium aerogenes. (Number of Fissions in Successive 5-Minute Intervals)

Time interval No.....	2	3	4	5	6	7	8	9	10	11	12	13	14
109 fastest cells.....	0	3	7	18	14	36	10	10	4	2	1	0	4
48 slowest ".....	1	2	9	11	2	4	5	2	9	3	0	0	1

TABLE V

Variability of Budding Times in Yeast. (Percentages of Budding Cells)

Time interval No.....	10	11	12	13	14	15	16	17	18	19	20	21
Series A 224 data.....	2.7	—	—	0.9	—	2.7	6.3	19.6	21.9	13.0	9.4	8.5
" B 194 ".....	—	—	—	—	—	—	—	—	—	—	—	2.1
Time interval No.....	22	23	24	25	26	27	28	29	30	31	32	33
Series A.....	1.8	0.9	0.9	2.2	4.0	2.7	2.7	—	—	—	—	—
" B.....	11.4	8.2	16.5	19.6	10.8	8.7	1.1	3.1	—	1.1	1.1	1.1

bacteria which corresponds to the requirement that the spread of variation is relatively smaller as the number of genes increases. There is no proof that a yeast has more genes than *Bacterium aerogenes*, but it appears probable. The variation between fast and slow growing cells is not inherent, but merely a matter of chance, as the theory demands.

It might be claimed, on the other hand, that the experiments do not contradict necessarily the other assumption that uniform cells multiply at the same moment, and that any differences in growth rate are caused

by differences in the cells or in environment. However, it seems that the great differences found in the growth rates of sister cells lying side by side in the same medium can hardly be accounted for in this way.

Chemical Interpretation of the Variability of the Growth Rate

The above discussion intends to show that what we ordinarily call variability of growth rate is not variability in the common sense of the word, but the result of the working of chemical laws. For the sake of simplicity, the genes have been supposed to be the simplest units of the chromosomes. This assumption is not necessary. The calculation is just as correct if we assume each gene to consist of a number of molecules, which, owing to their peculiar properties, shall be called gene-type molecules. As soon as we designate the letter g to mean the number of gene-type molecules per cell, and not the number of genes, the formula for the variability includes no assumption about the size of genes and the number of molecules in one gene.

Several assumptions have been made in this discussion which might be doubted. The entire deduction is based upon the assumption that in uniform cells, under uniform conditions, the molecules in the various cells react as if all cell contents formed a continuous medium. This assumption cannot be proved. It can only be stated that the order of death of bacteria is in agreement with this assumption. If this can be shown to be definitely wrong, then the entire deduction is wrong.

It has been further assumed that the reaction leading to the doubling of the gene-type molecules is monomolecular. This makes the calculations of the formulas easy. But if the reactions were of a higher order, the general principle of the "order of growth" would not be altered; only the formula and the curves would become much more complicated. There would be still a great difference in time between the first and the last completion of the doubling of all genes.

It has further been assumed that a cell divides as soon as all genes are doubled. Originally, the term "cell division" was just used as a simpler, shorter term for the doubling of all genes in one cell, but when we come to experimental proofs, it assumes a more definite meaning. If it takes 2 hours for all genes in one cell to double, and then 20 minutes longer before cell division can be actually observed, the frequency for the experimental order of growth lags 20 minutes behind the calculated curve. This inaccuracy does not change the type of curve, nor the principle of the "order of growth." But it prevents drawing far-reaching conclusions.

It has also been assumed that all gene-type molecules divide at the same rate. There is no evidence to prove or disprove this. If the rates of division were very different, this would make the problem much more difficult mathematically but

would not affect the principle under discussion, and would still give a large and experimentally measurable range of variability.

One further assumption is implied, namely, that a gene-type molecule, after having doubled, "waits" for all others to double, and does not start to double again until the chromosomes have doubled. Otherwise one gene might double a second time before another has doubled for the first time, and the balance of chromosomes would be upset. There must be some regulatory mechanism in the cell to prevent this. The nature of this mechanism is absolutely unknown. This regulation must exist in the cell even if the theory here presented should prove to be wrong.

The mathematical calculation had to be over-simplified to bring out the principle that variability of the growth rate is a chemical necessity. Of the many assumptions made for mathematical treatment, only the application of the mass law to molecules in a number of uniform cells is essential for the theory. This is the same assumption which the author has used to account for the differences in the order of death between bacteria and larger organisms (Rahn, 1929, 1930, 1931). All other assumptions do not affect the principle of the theory, but only the ease and accuracy of its mathematical formulation.

It has already been pointed out above that with unicellular organisms, the relative spread of variation (relative to the average time required) is largest with the simplest organisms. This might be used as a means to compute the number of gene-type molecules in unicellular organisms if we had very accurate data on the variability of the growth rate, and if cell division would take place at the same moment when the last gene has doubled.

We have to take the ratio of the times required to reach two definite points of the variability curve. We shall choose the moment when 10 per cent of the cells have doubled, and the moment when 90 per cent of the cells have doubled. The general equation was

$$P = (1 - q^t)^s$$

In the first case, we have $P = 0.1$; in the second case,

$$P = 0.9$$

The above formula gives $P^{\frac{1}{s}} = 1 - q^t$

$$q^t = 1 - P^{\frac{1}{s}}$$

$$t = \frac{\log \left(1 - P^{\frac{1}{s}} \right)}{\log q}$$

$$t_{10} = \frac{\log \left(1 - 0.1^{\frac{1}{g}} \right)}{\log q}$$

$$t_{90} = \frac{\log \left(1 - 0.9^{\frac{1}{g}} \right)}{\log q}$$

The ratio between these two times is independent of the growth rate, q :

$$\frac{t_{90}}{t_{10}} = \frac{\log \left(1 - 0.9^{\frac{1}{g}} \right)}{\log \left(1 - 0.1^{\frac{1}{g}} \right)}$$

It is difficult to determine g from this equation, but we can easily compute the ratio for any definite g . We find:

$$\text{for } g = 10, \quad r = \frac{t_{90}}{t_{10}} = 2.87$$

$$\text{for } g = 100, \quad r = 1.83$$

$$\text{for } g = 1000, \quad r = 1.58$$

The difference between the last two ratios is so small that it seems rather hopeless to determine the number of gene-types by this method if the number is high, considering also the inaccuracy in determining experimentally the order of growth. For organisms with less than 100 genes—if such organisms should exist—this method might suffice for an approximation provided that completely homogeneous material can be obtained for the purpose. Any variability in the organisms or in the environment will tend to increase t_{90} and to decrease t_{10} , i.e., it will increase the ratio $\frac{t_{90}}{t_{10}}$ and make the number of genes appear smaller than it really is.

The three sets of data on *Bacterium aerogenes* in Table III give the following values for $\frac{t_{90}}{t_{10}}$: 1.95; 1.85; 1.65.

From the two yeast experiments, this quotient assumes the values, 1.46 and 1.28. While we could draw no conclusions regarding the number of gene-type molecules per cell, we might conclude that yeast has a larger number of genes than *Bacterium aerogenes*.

There is one more agreement between theoretical calculation and experiment. Following a suggestion by Crozier (1931, footnote p. 20), it seemed probable that the skew of the theoretical frequency curves might be caused by the orderly gradual change of the velocity of

growth, or of the velocity of the reaction of the gene-type molecules. In this case, a normal, *i.e.*, symmetrical frequency curve should be expected if the number of organisms multiplying per time unit is divided by the time unit before being plotted against time. In the theoretical cases as well as in the experiments with bacteria and yeast, the frequencies thus plotted proved to be quite symmetrical.

Growth Rates of Multicellular Organisms

With multicellular organisms, only the time required for several consecutive cell divisions can be measured, and it is not certain that this number is the same in each individual. For example, it seems doubtful to the author that all insect larvae of one species have the same number of cells. Besides, there is, with most higher organisms, a considerable amount of specialization of the cells, and different types of cells will probably multiply at a different rate. The simplest case where all cells are at least functionally alike is that of bacteria colonies. This is the only case that can be treated mathematically. Even there, we have to make the assumption that the average growth rate remains constant which will not be correct for the later stages of development.

A mathematical treatment may be possible, but the author did not succeed. However, a purely empirical calculation of the variation of the time required to complete three or ten cell divisions is simple. Table I has shown that for $q = 0.1$ and $g = 100$, the frequencies of cell division for the first five time units are: 0; 36.6; 52.78; 9.62; 0.90. Each of the cells born at these times has the same chances again during the second cell division, and it can be easily computed how the fission times of the offspring of the first 36.6 per cent will vary. The 52.78 per cent of the third time interval again multiply according to the same probability curve, and so do the 9.62 per cent and the 0.9 per cent of the other two time units. In this tedious, but simple way, the frequency distribution for any number of generations can be computed, as Table VI shows, and Table VII gives the result in per cents for ten generations of the above example. By these ten divisions, the cells have multiplied 1024-fold.

If an organism existed which consisted of ten generations = 1024 cells of uniform composition, the last row of Table VII would show the expected variation of time required for the development of this organ-

TABLE VI
Calculated Number of Cells Completing the 1st, 2nd, 3rd, 4th, and 5th Generation during Successive Time Units ($g = 100$, $q = 0.1$)

Time unit	No. of cells dividing per time unit																			Total cells
	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th	13th	14th	15th	16th	17th	18th	19th	
Generation I.....	0	366	528	96	9	0	0	0	0	0	0	0	0	0	0	0	0	0	1,000	
".....	0	0	0	268	776	698	216	38	4	0	0	0	0	0	0	0	0	0	2,000	
".....	0	0	0	0	0	0	196	852	1,384	1,050	404	98	16	0	0	0	0	0	4,000	
".....	0	0	0	0	0	0	0	142	834	1,954	2,400	1,688	732	208	42	0	0	0	8,000	
".....	0	0	0	0	0	0	0	0	0	104	760	2,342	3,988	4,150	2,830	1,292	422	100	12,16,000	

$$(g) = 100,$$

VARIABILITY OF GROWTH RATE

TABLE VII

of the Times Required by Unit
q = 0.1)

[illegible]

ism from a single cell. Table VII and Fig. 4 indicate that the frequency curve becomes flatter as the number of generations increases, but it also shows that the relative spread of variation decreases.

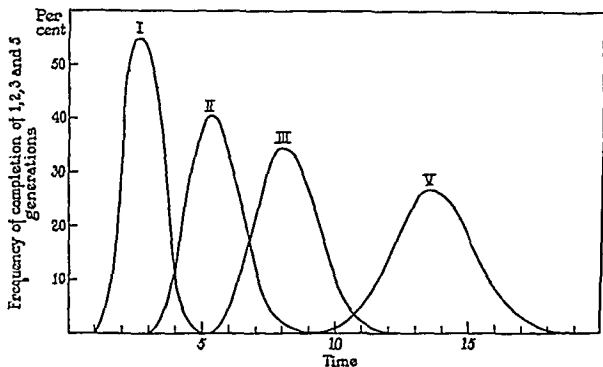


FIG. 4. Percentage of cells completing their 1st, 2nd, 3rd, and 5th division in successive time units.

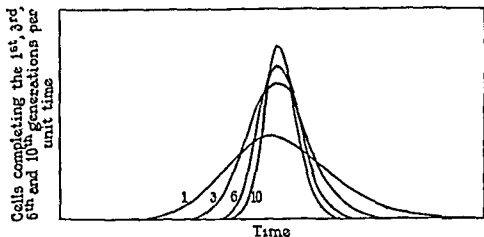


FIG. 5. Percentage of cells per unit time completing 1, 3, 6, and 10 generations, drawn to equal modal distances.

This is demonstrated graphically in Fig. 5 which presents four of the series from Table VII in such a way that the modes of all of them coincide. This required a different scale on the abscissa for each curve;

to keep the area of the curves uniform, the ordinates were enlarged in the same proportion as the abscissae were shortened. The figure shows very plainly how the relative variation decreases as the size of the "organism" increases from 1 to 1024 cells. It also shows that with an organism of this size, the variability of the growth rate should still be experimentally measurable.

An experiment with bacteria colonies verified this expectation. A number of nutrient agar plates were flooded with a very young culture of *Bacterium aerogenes* which had been transferred repeatedly at 3 hour intervals. The cells remaining on the agar surface after pouring off the excess liquid developed at 30° into colonies. At four different intervals, some of the plates were treated with formaldehyde to prevent further growth. The diameter of 100 colonies was measured for each

TABLE VIII
Variation in Diameter of Bacteria Colonies of Four Different Ages

Intervals, some of which show further growth. The diameter

TABLE VIII

Variation in Diameter of Bacteria Colonies of Four Different Ages

Age of colonies	One division of the scale equals	Percentage of colonies having the following diameter of the scale										
		30	40	50	60	70	80	90	100	110	120	
6½ hrs. old.....	0.8 μ	—	1	5	6	24	31	19	8	3	3	
9¼ hrs. old.....	8.0 μ	1	9	20	32	21	11	—	—	—	—	
13½ hrs. old.....	23.3 μ	—	—	4	8	46	23	16	2	—	—	
16½ hrs. old.....	46.6 μ	2	—	11	53	22	9	3	—	—	—	

of development, and the results arranged by frequency of development, and the results arranged

of the four stages of development, and the results arranged by frequencies, are given in Table VIII.

The scales have been chosen so that the entire range of variability can be compared in one table, and it is evident that with increasing age, the relative range decreases. That is all that could be proved by this experiment, and it agrees with the theoretical deduction of the preceding pages.

The general principle that increase of the number of generations, i.e., increase of the number of cells of an organism, decreases the relative range of variability is quite evident from a number of data on multicellular organisms. Fig. 6 shows a few such data of different organisms drawn in the same manner as Fig. 5, namely so that all modes coincide. The data used are the gestation period of rabbits,

from 200 data kindly provided by Mr. R. B. Hinman of the Animal Husbandry Department of Cornell University, and the gestation period of cows, after data from Wing (1899). For comparison, one yeast experiment from Table V, and the last group from Table III of *Bacterium aerogenes* are included.

Data on the hatching times of insect eggs, also on other stages of insect development, have been furnished by Sanderson and Pears (1913), Parker (1930), and many others, but the eggs do not represent single

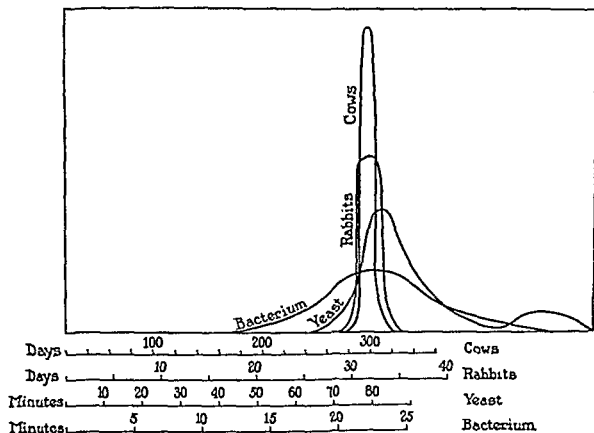


FIG. 6. Variability of the growth rate of a bacterium and a yeast, and of the gestation period of rabbits and cows, all drawn to equal modal distances.

cells, and therefore do not fit into Fig. 6. Most of the frequency curves plotted from these data are skewed to the left, but there are a number of exceptions. The curves on the gestation period of cows and rabbits, are practically symmetrical. Data on the "gestation period" of insects could not be found.

The question arises whether this difference in the growth rate of identical cells, if it is due to chemical laws, should be called "variability." It seems to be the practice of limiting this term in technical

language to differences in the composition of the organism which are too slight to be detected by other means. When the cause of different behavior of similar organisms is known, it is not usually termed "variability."

In this case, the cause of "variability" seems to be the mass law. The cause is known, but it cannot be eliminated experimentally. We cannot make 100 uniform bacteria cells divide all at the same time any more than we can make all sucrose molecules of a sugar solution invert at the same moment. In this respect, the cause of variation of the growth rate is different from the causes of all other variations.

SUMMARY

The general belief that uniform cells under uniform conditions will all multiply at the same moment implies that the smallest units of the chromosomes, *i.e.*, either the genes or the molecules of which the genes are composed, all double at exactly the same moment in all cells.

Since the doubling of chromosomes is a synthetic chemical process, it seems more probable that it would follow chemical laws. With the assumption that the corresponding molecules in a number of uniform cells obey the mass law in their process of doubling, a definite order in the multiplication of identical cells is established which can be formulated mathematically for the simplest case. This is the same assumption which the author has used to account for the differences in the order of death between bacteria and higher organisms.

This theory demands a great variability of the growth rate of uniform cells, so great that it must be experimentally measurable even for cells with a million molecules to the chromosome.

The theory demands further that the frequency curve of cell divisions plotted for successive time intervals, be skewed to the left, and that the relative range of variation become smaller as the number of genes or gene-type molecules increases.

Experiments on the growth rate of *Bacterium aerogenes* and *Saccharomyces ellipsoideus* showed regularly a frequency curve skewed to the left. The yeast had a relatively narrower range of variability than the bacterium.

Even with multicellular organisms, theoretical calculations show a range of variation of the growth rate from the egg cell which should still be measurable though it decreases relatively with the number of cells produced. An experiment on the size of bacteria colonies at different ages of development agreed with the theory.

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ELECTROKINETIC PHENOMENA

V. A SMALL BUT CONSTANT SOURCE OF ERROR IN MEASUREMENTS OF VISCOSITY

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During the course of a conversation with Professor Grinnell Jones, we learned of certain inexplicable data obtained during the measurement of the viscosity of solutions of low ionic strength. In particular, variations in the time necessary for a given volume of distilled water to flow out of the (Ostwald type) viscosimeter, as well as certain anomalous features of the viscosity-concentration curves, suggested to us the possibility that some of the difficulties encountered were due to the electric charge on the quartz wall of the viscosimeter. During the laminar flow of a liquid of volume V , under hydrostatic pressure P , past a quartz surface, a streaming potential E , is set up across the capillary of the viscosimeter,

$$E = \frac{\zeta P D}{4 \pi \eta \kappa}; \quad (1)$$

ζ = electrokinetic potential or the potential between the surface and the liquid; D = dielectric constant of the medium; η = viscosity of the liquid, κ = specific conductance of the liquid.* A difference of potential between capillary wall and adjacent solution must exercise a retarding effect on the charged molecules of water near the quartz surface. Thus it must take a longer time for the charged viscosimeter to drain itself than if the wall had been uncharged.

We may calculate as follows the order of magnitude of the error which can be introduced by this retarding effect for the size of capil-

* When the radius of the capillary becomes very small, surface conductance must be considered.

laries in common use with dilute electrolytes and distilled water. Let V_F be the volume of outflow of the liquid per second from an uncharged capillary of radius r . According to Poiseuille,

$$V_F = \frac{\pi P r^4}{8 \eta l}. \quad (2)$$

If the wall is charged, a volume v is retarded so that from a charged capillary a volume $(V_F - v)$ will really flow during the time that the volume V_F required for outflow. Experience shows that

$$v < < V_F,$$

so that $\frac{v}{V_F} = \frac{v}{V_F - v}$, very nearly.

The ratio $\frac{v}{V_F}$ gives a good idea of the fraction of the total time that is required additionally for the charged capillary. It is approximately correct that the relationship between the streaming potential in the capillary, E , and the retarded volume, v , is given by the relationship

$$v = \frac{r^2 \zeta E D}{4 \eta l}. \quad (3)$$

The ratio $\frac{v}{V_F}$ is obtained from (2) and (3),

$$\frac{v}{V_F} = \frac{2 \zeta E D}{\pi P} \cdot \frac{1}{r^2} = \frac{K}{r^2},$$

where K is a constant.

Krulyt gives the following data for the streaming potentials in a glass capillary with KCl solutions.

KCl		E ($P = 13.6$ cm. H_2O)	
Mols $\times 10^3$		Volts $\times 10^3$	
0		350	
50		102	
100		57	

Since we are concerned with the order of magnitude of a phenomenon in very dilute salt solutions where E and ζ vary very much from one instance to another, it is sufficient to take a simple illustrative case where $E = 200 \times 10^{-3}$ volts and $\zeta = 200 \times 10^{-6}$ volts (electrostatic units) to evaluate K . The value of K then amounts to $\frac{10^{-9}}{2}$, approximately; $\frac{K}{r^2}$ can be obtained from the following table:

r	r^2	$\frac{2K}{r^2}$
cm.	cm ² .	
0.041	0.0017	1×10^1
0.031	0.0010	1×10^{-1}
0.021	0.0004	1×10^{-2}
0.01	0.0001	1×10^{-3}
0.10	0.01	1×10^{-7}

For capillaries 1×10^{-4} cm. in radius the error is as much as 10 per cent. Professor Jones' capillary was of the order $r = 0.02$ cm. The error due to this effect for capillaries of this radius is of the order $\frac{1}{800,000}$. Professor Jones measures time to one part in 50,000. The use of capillaries less than $r = 0.01$ cm. should be avoided as the retarding effect then increases most rapidly; thus, for $r = 0.01$ it is very near to the limits of experimental error.

Dr. Lars Onsager and Dr. K. C. Cole have been kind enough to discuss this problem in detail with me and to obtain by other methods of calculation the same order of magnitude of the error involved. Dr. Onsager points out that with liquids of very low conductance, the effect is proportional to the square of ζ . This is given in the expression, $\frac{v}{V_F} = \frac{8h^2\eta}{\kappa r^2}$, where $h = \frac{D\zeta}{4\pi\eta}$, which Drs. Onsager and Cole have been able to obtain.

VARIATION OF THE ELECTRIC RESISTANCE OF PLANT TISSUES FOR ALTERNATING CURRENTS OF DIFFERENT FREQUENCIES DURING DEATH

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The study of the electric resistance of living cells, in relation to variation of frequency of alternating current, developed during the last 20 years by Höber (1910), Gildemeister (1919), Philipppson (1920), Waterman (1922), Fricke (1923), McClendon (1924), Fricke and Morse (1925), Blinks (1928), and Remington (1929), has been used chiefly in determining physical characteristics of various tissues such as muscle, liver, skin, red corpuscles, plant cells and in special investigations on subjects such as the resistance of malignant tumors; but such problems of general physiology as growth or death, in relation to variation of frequency, remain almost untouched. Therefore, since the method seemed promising, I undertook the present study of the variation of the resistance of a plant tissue which was injured or killed by heating or freezing or by the action of poisonous substances.

Apparatus, Material, and Working Methods

The measurements were made with the high frequency bridge described by H. Fricke (1925a, 1925b), and Fricke and Morse (1925, 1926). The frequencies used vary from 0.5 to 1024 kilocycles.

The conductivity cell is of the same kind as that described by Fricke (1926). The tissue is fastened in the hole of an isolating diaphragm of celluloid which divides into two parts a solution of KCl in which the platinized platinum electrodes are immersed. The hole is cylindrical and measures 1/4 of an inch in diameter and 1/8 of an inch in length.

The piece of tissue to be placed in the hole is cut with a cylindrical borer which has an inside diameter of 0.266 inch. After being fastened in the hole, both ends of the piece are shaved with a razor blade. In experiments in which the tissue is to be injured it is cut 1/2 inch in length and a borer with an inside diameter of 0.400 inch is used instead of the one referred to above. Then after being treated

it is recut to fit in the hole. An exact fit is impossible if the tissue is cut to size before treating, because of the contraction or softening which takes place.

When a tissue is placed in a salt solution its resistance may change continuously. If the external solution is concentrated the resistance decreases, if dilute it increases, while an intermediary concentration gives an almost constant resistance. Several solutions with different concentrations were tried in my experiments. KCl M/25 was selected as giving the best results during the 30 minutes necessary for the readings.

As the material for experimentation, I chose *Ambrosia trifida*. I used plants young enough and pieces close to the tip (3 to 20 cm.) in which the pith had not yet begun to show a white opaque appearance and where there was little lignification. The pieces used in the same experiment were cut from the same stem and as near to each other as possible. They were always cut in such a way that the direction of the current was parallel to the axis of the stem.

The tissue was heated by immersion in a KCl M/25 solution previously warmed to the desired temperature. Freezing was performed in a cylinder of thin brass buried in carbon dioxide snow. After having been warmed or frozen, the tissue was, of course, brought back to the usual temperature for the electric measurements. Injuring with chemicals was produced by immersion. Tissues were then washed rapidly and put in the conductivity cell.

DATA

Resistance of Tissue Injured by Heating (50°), Boiling, Freezing (CO₂) and by the Action of Ether and Alcohol (95 Per cent)

(Frequency in kilocycles; Resistance in ohms of a cube 1 cm. on each side)

Frequency	Normal tissue	2 min. 50°	4 min. 50°	6 min. 50°	Boiled tissue	10 sec. CO ₂	10 min. ether	2 min. alcohol
0.5	871	602	325	263	157	508	428	395
1	862	565	324	263	154	518	425	391
2	840	552	322	258	153	521	424	386
4	823	554	306	262	148	500	423	385
16	722	518	298	253	143	471	393	361
32	549	499	286	250	143	445	343	342
64	355	375	269	239	143	380	262	301
128	253	299	228	237	142	282	193	273
256	168	212	206	233	136	196	130	234
512	125	163	194	221	129	134	94	199
1024	90	113	179	214	130	102	81	198

DISCUSSION

1. At low frequencies the resistance decreases with the increase of the injury until it reaches a minimum which is the resistance of dead

tissue (this effect has been extensively studied by Osterhout). At high frequencies the resistance of dead and living tissues is nearly the same and its value approaches that of dead tissue with low frequency.

2. The preceding facts are in agreement with the idea that the electrical resistance of tissues under experiment is due mostly to the sur-

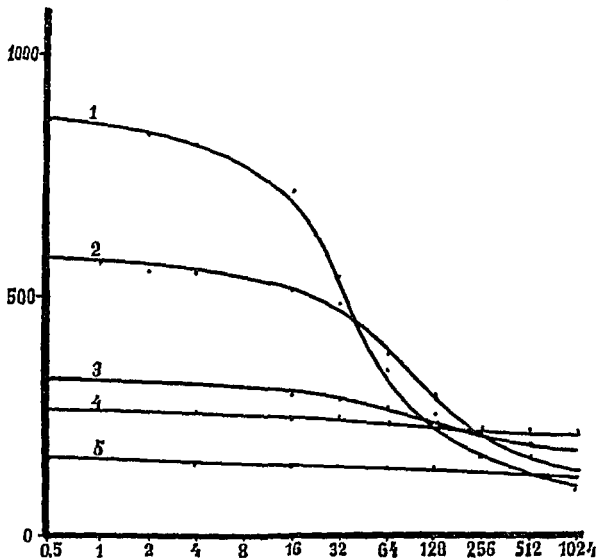


FIG. 1. Resistance of the plant tissue (1) under normal conditions, (2) heated 2 minutes at 50°C., (3) heated 4 minutes at 50°C., (4) heated 6 minutes at 50°C., and (5) boiled 20 minutes. Abscissae: Log of frequencies in kilocycles. Ordinates: Resistances in ohms.

faces of the cells. It is well known that injury and death are accompanied by a more or less complete destruction of these surfaces. The decrease in resistance at low frequencies seems to correspond to the degree of this destruction. On the other hand it is well known that resisting films have less effect the higher the frequency of the current,

so the results at high frequencies also agree with the preceding assumption.

3. Is the drop of resistance following the injury due to an increasing number of damaged cells or to an increasing permeability of all the cells? This cannot be decided by my experiment.

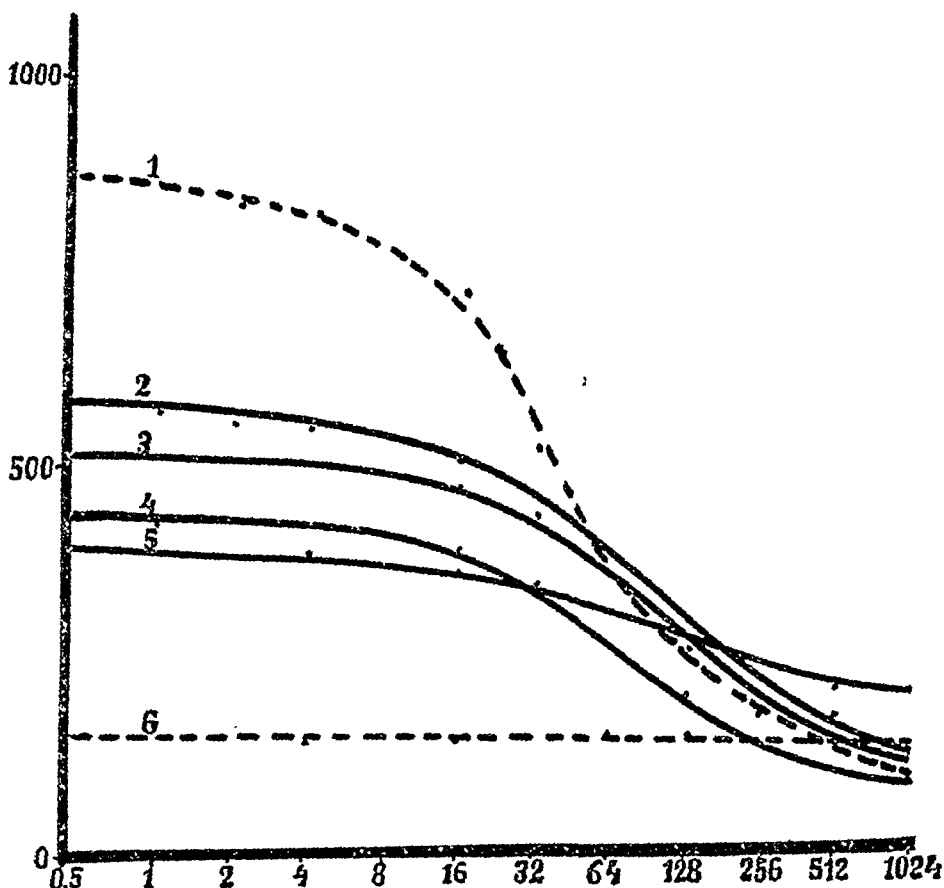


FIG. 2. Resistance of the plant tissue (1) under normal conditions, (2) heated 2 minutes at 50°C., (3) plunged 10 seconds in solid CO_2 , (4) plunged 10 minutes in ether, (5) plunged 2 minutes in alcohol (95 per cent), and (6) boiled 20 minutes. Abscissae: Log of frequencies. Ordinates: Resistances in ohms.

4. It will be noticed that the difference of time of exposure necessary to produce a complete drop of resistance (until the resistance of dead tissue is reached) and to produce any other sign of death is enormous. A few minutes at 50°C. are enough to make the resistance

drop to its limit, whereas hours are necessary to produce any sign of death with usual indicators or to change the turgor or to make noticeable the characteristic color of killed plant tissue.

5. An injury by ether behaves in the same way as an injury produced by heating or freezing, both processes showing parallel curves (Fig. 2, Curves 2, 3, and 4). Alcohol (Fig. 2, Curve 5) shows a somewhat different curve.

I express my gratitude to Dr. H. Fricke, Dr. W. Osterhout, and Dr. R. Harris for their valuable suggestions.

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ON THE PHYSICAL NATURE OF "CYTOTROPISM" AND ALLIED PHENOMENA AND THEIR BEARING ON THE PHYSICS OF ORGANIC FORM

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In 1894 W. Roux (24) described a phenomenon, to which he gave the name of "Cytotropism," and which is manifested by forces of attraction (and sometimes of repulsion), between isolated blastomeres of a frog's blastula. Roux strongly emphasized the importance which "Cytotropism" may have in the mechanism of determination and development of the form of organisms. As to the physical nature of the forces involved, Roux suggests and discusses the possibility, that they may be of the same nature as the forces observed in the phenomenon of "chemotaxis." To the author's knowledge nothing, however, has been done towards a theoretical study of the physical nature of chemotaxis, except a paper by L. Rhumbler (23). According to Rhumbler, the positive chemotaxis is due to the presence, in the surrounding medium, of substances which lower the surface tension of the cell. If the concentration of those substances around the cell is non-uniform, the cell will have a smaller surface tension on the side turned towards the higher concentration, and this will result in the formation of an amoeboidal protrusion in this direction, with the result that the cell will move towards places of larger concentration. The correctness of this conclusion is illustrated by Rhumbler on non-living models.

Any kind of mechanical forces between cells may play an important rôle in the determination of the form of the tissue composed of those cells (6). If we wish, therefore, to attempt a physical explanation of the organic form, we necessarily must first of all study the various physical forces, which *may* come into play. A physical theory of

organic forms must be preceded by a general mathematical theory of the forces which may play a part in the development of these forms.

The purpose of this article, which is intended to be the first step of a theoretical study on the physics of organic form, is to show that mechanical forces of various kinds between cells must be expected on a rather general physical basis, and do not require any special assumptions for their explanation.

We wish, however, to emphasize strongly that no claim is made in the following that the forces which we shall study actually explain the phenomenon of "cytotropism." The latter may or may not be due, at least partly, to forces of such a nature as those discussed here. But if we wish to have a theoretical insight into the possible mechanism of a complicated phenomenon, we should, following the usual procedure of theoretical physics, first make a mathematical study of some simpler, idealized, but possible cases. And whatever the complete explanation of "cytotropism" may finally happen to be, it is of importance for the physiologist to know that similar forces may be due to very general physical phenomena.

So far as phenomena of chemotropism are concerned, they, in their complexity, almost certainly fall out of the scope of the following considerations, since they mostly deal with forces which tend to orient highly complex organisms, which even in the absence of those forces possess the property of spontaneous locomotion (2, 8, 9, 10, 11, 16, 17, 18); whereas the forces studied below cause the movement of otherwise immobile, comparatively simple systems. In this respect the phenomena of cytotropism seem to be of a simpler nature than the more general phenomena of chemotaxis, and may fall within the scope of the present investigation.

I

Suppose that a small drop of a substance, A , is immersed in another liquid, B , immiscible with A . Let B contain in a dilute* solution a

* Qualitatively the following results hold true for any concentrations. However only for dilute solutions do we possess explicit expressions for the various thermodynamical functions and therefore only for those are we able to derive quantitative formulae. The order of magnitude of all the effects concerned will be however the same for dilute and concentrated solutions.

substance C , which we suppose to be soluble both in A and in B . We do not make any other assumptions as to the physicochemical nature of either A , B or C . The concentrations of C in A and B will in general be different. Denoting the concentration of C in A by c' the concentration of C in B by c , the heat of solution of C in A by λ' , the heat of solution of C in B by λ , we shall have: (7, p. 462)

$$\frac{c'}{c} = e^{-\frac{(\lambda' - \lambda)}{RT}} \quad (1)$$

R denoting the gas constant and T the absolute temperature. We have

$$c' \geq c$$

for

$$\lambda' \leq \lambda. \quad (2)$$

λ , and correspondingly λ' , is defined as the work which must be done in order to bring one gram-mol of C into the corresponding solution. Hence if λ is negative, the process of solution is exothermic.

Next let us consider the case, that C is distributed in B non-uniformly. Let A first be situated in a region where the concentration c has a value c_1 and let us then bring the drop A into a region with the concentration c_2 . The concentration c' of C in A will have changed from αc_1 to αc_2 , α being defined by:

$$\alpha = e^{-\frac{(\lambda' - \lambda)}{RT}} \quad (3)$$

Let us consider the change in the free energy of the whole system: "drop + surrounding medium" which takes place as a result of such a change of place of A . The free energy of the drop will be a function of the concentration $c' = \alpha c$, as well as of the total pressure and temperature, the latter two factors being kept constant. Let this free energy be equal to F_{1A} for $c = c_1$ and to F_{2A} for $c = c_2$. The process of transferring the drop A from 1 to 2 may be made in the following steps (Fig. 1): we imagine the drop to be taken away from 1, and at the same time an equal volume of B taken away from 2, and then the two exchanged their places. In other words, the transfer of the drop A

from 1 to 2 is necessarily accompanied by a transfer of a similar drop of the substance B from 2 to 1. No other changes occur in the system. The free energy of the corresponding volume of B is also a function of the concentration c of C . Let this be F_{1B} for $c = c_1$, and F_{2B} for $c = c_2$.

Before the transfer the free energy of the system was equal to

$$F_{1A} + F_{2B} + F_o, \quad (4)$$

F_o denoting the free energy of the rest of the solution. After the transfer of A from 1 to 2, the free energy equals

$$F_{2A} + F_{1B} + F_o. \quad (5)$$

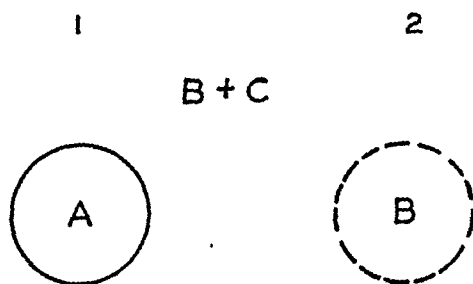


FIG. 1

Hence the difference between the initial and the end state:

$$\Delta F = F_{2A} + F_{1B} - (F_{1A} + F_{2B}) = (F_{2A} - F_{2B}) - (F_{1A} - F_{1B}). \quad (6)$$

If $\Delta F < 0$, work is gained by moving A from 1 to 2, and according to the principles of thermodynamics, this work gained equals ΔF , regardless of the path in which we moved the drop (19).

But this means that there is a force, which tends to move the drop A from 1 to 2, and that this force is derived from a potential. Since both F_A and F_B are functions of c , which in its turn is a function of the coordinates x, y, z , we may write Equation 6 as follows:

$$\Delta F = [F_A(x_2, y_2, z_2) - F_B(x_2, y_2, z_2)] - [F_A(x_1, y_1, z_1) - F_B(x_1, y_1, z_1)] \quad (7)$$

and since, as we said, the work gained is independent of the path along which A is moved from the point x_1, y_1, z_1 to x_2, y_2, z_2 , it follows that the force which tends to bring A from 1 to 2 is given by

$$f = - \text{grad} (F_A - F_B) = - \frac{d(F_A - F_B)}{dc} \text{grad } c. \quad (8)$$

If

$$\frac{d(F_A - F_B)}{dc} < 0,$$

the force tends to move the drop towards places of higher concentration of C , otherwise — in the opposite direction. Such forces exist quite generally, except when either c is constant or else

$$\frac{d(F_A - F_B)}{dc} = 0.$$

Let us now find an explicit expression for the force f , which will enable us to calculate at least its order of magnitude.

The free energy of the drop A is composed of two parts: the free energy of the solution of C in A , and the surface energy: $F_A = F_A' + F_A''$

The first part is equal to:

$$F_A' = c \frac{\alpha V}{M_C} \left(\lambda' + \frac{3}{2} RT - \varphi_1 T + RT \lg \frac{\alpha M_A}{\delta_A M_C c} \right) + \text{Const.}, \quad (9)$$

where the different letters have the following meanings:

M_A — molecular weight of A

M_C — “ “ “ C

δ_A — density of A

V — volume of the drop

and where the constant does not contain c .

Equation (9) is obtained from

$$F = U - T\Phi$$

(U — total energy, Φ — entropy), if for U and Φ

PHYSICAL NATURE OF "CYTOTROPISM"

duce the equations (209) and (213) of Planck's Thermody-
19), and if we remember that for n_0 we must substitute

$$n_0 = \frac{V\delta_A}{M_A}$$

$$: n_1 \quad n_1 = \frac{c'V}{M_C} = c \frac{\alpha V}{M_C}.$$

(10)

second part is equal to

$$F_A'' = \gamma(c)S$$

;) being the surface tension which is in general a function of c , and
— the total surface of the drop. For a spherical drop of a radius
we have

$$V = \frac{4}{3}\pi r_0^3, S = 4\pi r_0^2, S = \sqrt[3]{36\pi} V^{\frac{2}{3}}$$

(11)

As regards F_B , this is composed of one part only, corresponding to
equation (9), since there is no physical interface between the drop and
the surrounding medium. Hence:

$$F_B = c \frac{V}{M_C} \left(\lambda + \frac{3}{2} RT - \varphi_1 T + RT \lg \frac{M_B}{\delta_B M_C} c \right) + \text{Const.}, \quad (12)$$

Therefore

$$F_A - F_B = c \frac{V}{M_C} \left[\alpha \lambda' - \lambda + \frac{3}{2} (\alpha - 1) RT - \varphi_1 (\alpha - 1) T + \right. \\ \left. (\alpha - 1) RT \lg c + \alpha RT \lg \frac{\alpha M_A}{\delta_A M_C} - RT \lg \frac{M_B}{\delta_B M_C} \right] \\ + \gamma(c)S + \text{Const.}, \quad (13)$$

From equations (8) and (13) we obtain:

$$f = - \left\{ \frac{V}{M_C} \left[\alpha \lambda' - \lambda + \frac{5}{2} (\alpha - 1) RT - \varphi_1 (\alpha - 1) T + \alpha RT \lg \frac{\alpha M_C}{\delta_A M_C} \right. \right. \\ \left. \left. - RT \lg \frac{M_B}{\delta_B M_C} + (\alpha - 1) RT \lg c \right] \right. \\ \left. + S \frac{d\gamma(c)}{dc} \right\} \text{grad } c.$$

The last term in the braces represents the force due to the influence of C on the interfacial tension of $A-B$. If this term is very large as compared with the others, so that the latter may be neglected, equation (14) reduces to

$$f = - S \frac{d\gamma(c)}{dc} \text{ grad } c, \quad (15)$$

If the addition of C decreases the surface tension, we have

$$\frac{d\gamma(c)}{dc} < 0,$$

and in this case equation (15) shows that the force will act in the direction of increasing concentration. This is the force discussed by Rhumbler (23). For

$$\frac{d\gamma(c)}{dc} > 0,$$

the force would act in the direction of decreasing concentration. This case, however, is very unlikely to occur, for, as is known, for substances which *increase* the surface tension, this increase is usually very small, whereas a decrease of surface tension due to capillary adsorption may be quite considerable (5, p. 383). Hence if $\frac{d\gamma}{dc}$ is positive, it is usually very small, and the more complicated equation (14) must be used instead of equation (15).

Considering the other extreme, that C has practically no influence on γ , so that

$$\frac{d\gamma}{dc} = 0,$$

we see that the magnitude and direction of f is determined by the expression in the square brackets. This latter may be either positive or negative, depending on the choice of the various constants, in other words on the nature of the substances used. If this expression vanishes, f vanishes also. In general, however, it will be different from zero, and we thus see, that even in the absence of any specific action of the dissolved substances on the interfacial tension, a force will act

in a field of non-uniform concentration. If not only both λ and λ' , but also $\alpha\lambda' - \lambda$ are very large as compared with the other terms in the square brackets, then the direction of the force is determined by $\alpha\lambda' - \lambda$. If $\alpha\lambda' > \lambda$, the force acts in the direction of decreasing concentrations, otherwise in the opposite direction.

A few words may be said to help visualize the physical nature of the force which is due to the expression in square brackets of equation (14). Let both λ and λ' be negative and let $|\lambda| < |\lambda'|$. This means that when C is dissolved in either A or B , energy is liberated. In other words, there is a force of attraction between the molecules of A and C or B and C . The resultant force acting on a molecule of C

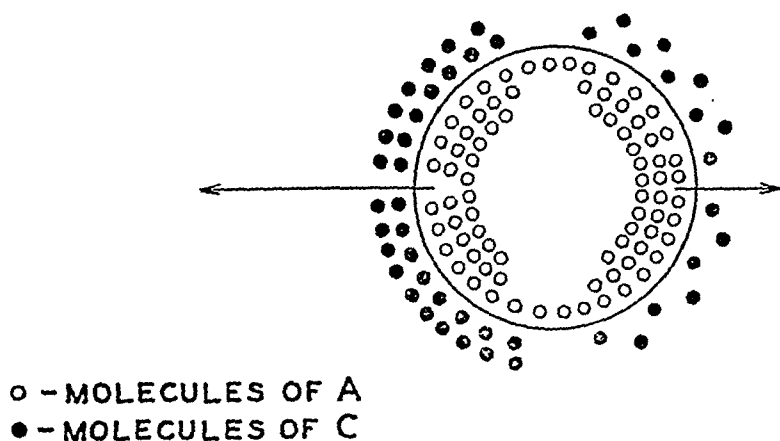


FIG. 2

dissolved in A is zero, because the molecule C is surrounded by molecules A from all sides. But there is a force of attraction between a molecule C (Fig. 2) dissolved in B near the surface of A , and the molecules of A . There is also a force of attraction between the molecules B and C , but the latter is smaller, since we assume $|\lambda| < |\lambda'|$. Hence the presence of the drop A tends to pull all molecules of C on the left side of A towards the right, or by the law of action and reaction, there is a pull on A from right to left. The amount of this pull is the greater, the more molecules of C are present around A . If the concentration of C is non-uniform, and greater to the left of A , the pull on this side of the drop will be larger (Fig. 2), and the drop will move in the direction of higher concentration.

Thus a drop, or a cell of volume V , when placed in a solution of non-uniform concentration, will be subject to a force, given by equation (14). If several substances are dissolved in the surrounding medium, the total force will be obtained by taking the sum of all similar expressions for all dissolved substances. In special cases it may, of course, happen that the total force will vanish.

So far we have considered the drop as sufficiently small, so that we can speak of it being "in a region of concentration c ". If the drop is large, the concentration will be different at different points in the drop. But our results hold unchanged. By a similar line of argument, we may imagine the drop to be transported from one place to another, and effectuate this transportation by infinitesimal parts, into which we can subdivide the drop. We shall find thus, that the total force on the drop will be given by such an expression:

$$f = - \left\{ \int dV \frac{1}{M_C} \left[\alpha \lambda' - \lambda + \frac{5}{2} (\alpha - 1) RT - \varphi_1 (\alpha - 1) T + \alpha RT \lg \frac{\alpha M_A}{\delta_A M_C} \right. \right. \\ \left. \left. - RT \lg \frac{M_B}{\delta_B M_C} + (\alpha - 1) RT \lg c \right] \right. \\ \left. + \int_S dS \frac{d\gamma(c)}{dc} \right\} \text{grad } c, \quad (16)$$

the first integral being extended over the whole volume of the drop, the second over its surface. We may say, that at each point in the drop a force is acting, equal at that point, per unit volume, to the integrand of the first integral times $\text{grad } c$, and at each point of the surface a force is acting, equal per unit area to

$$- \frac{d\gamma}{dc} \text{grad } c.$$

If we consider the most general case, that the substance which causes the above forces are either produced in the drop itself, and diffuse outside, or are destroyed in the drop, and supplied from outside, we shall find that each drop is surrounded by a field of non-uniform concentration, and two similar drops will therefore either attract or repel each other.

Let the substance C be produced inside the drop A at a constant rate $q \frac{gr.}{\text{min.}}$ per cm.^3 . If we denote the coefficient of diffusion of C in A by D_i , then the equation governing the whole process is

$$D_i \nabla^2 c' = -q$$

or in polar coordinates, and on account of the spherical symmetry,

$$\frac{d^2 c'}{dr^2} + \frac{2}{r} \frac{dc'}{dr} = -\frac{q}{D_i}. \quad (17)$$

Outside of the drop, we have,

$$\frac{d^2 c}{dr^2} + \frac{2}{r} \frac{dc}{dr} = 0. \quad (18)$$

As regards the boundary conditions, they are as follows:

$$-D_i \frac{dc'}{dr} = h(c' - \alpha c)$$

and

$$-D_e \frac{dc}{dr} = h(c' - \alpha c) \quad (19)$$

for $r = r_0$, r_0 being the radius of the drop.

D_e is the coefficient of diffusion of C in B , and h is the permeability of the drop boundary. We take $(c' - \alpha c)$ and not simply $(c' - c)$, because in absence of any diffusion processes $c' = \alpha c$. Hence the amount diffusing through the boundary is proportional to $(c' - \alpha c)$, being zero for $c' = \alpha c$.

The system of equations is easily solved by a substitution

$$c' = \frac{u'}{r}; \quad c = \frac{u}{r}$$

and is in its final shape:

$$c' = \alpha c_0 + \frac{q}{3h} r_0 + \frac{\alpha q}{3D_e} r_0^2 + \frac{q}{6D_i} (r_0^2 - r^2) \quad (20)$$

$$c = c_0 + \frac{qr_0^3}{3D_e} \frac{1}{r}. \quad (21)$$

c_0 is the constant concentration of C in B , at infinite distance from the drop. We see that inside the drop the concentration decreases from the center to the periphery, outside of it it decreases with distance like $\frac{1}{r}$. If we neglect the difference of the heats of solution, that is if we put $\alpha = 1$, and if we assume that D_e is very large, we find $c = c_0$; that is, the outside concentration is not affected by the presence of the drop, and remains uniform. Whereas c' reduces in this case to expressions which we derived and used in several previous papers (21, 22). If the substance is not produced, but is absorbed in A , we obtain the necessary expressions merely by changing in equations (20) and (21) the sign of q . If in this case D_i is very large, we find

$$c' = \alpha c_0 + \frac{q}{3h} r_0 + \frac{\alpha q}{3D_i} r_0^2; \quad (22)$$

that is, the concentration inside the drop is uniform.

These expressions hold for an isolated drop, surrounded by B , the latter extending to infinity. For several drops at finite distances, the distribution of c at any point, whether outside or inside of one of the drops, is obtained by a mere superposition of the solutions holding for each individual drop, since our equations are linear. And then the forces may be computed, using equation (16). It may be mentioned that in cases when the last member in the square brackets of Equation 16, as well as the surface integral, are negligible, the force between two spherical drops of finite size varies as the inverse square of the distance between their centers.

It is, unfortunately, impossible to apply our formula directly to any experimentally known case, and this for two reasons: first, the forces occurring in cytotropism have never been measured, and second, we have no sufficient knowledge of the substances produced and absorbed by cells, and hence of the constants, which enter into our formulae. But we can make a rough estimate of the order of magnitude of these forces. Remembering that (19)

$$\varphi_1 = \frac{3}{2} R_1 g T + R_1 g R T - R_1 g p + k_1,$$

p being the pressure and k_1 the entropy constant, and that the heats of solution per gram-mol are all of the order of 10^{10} erg, we easily estimate that the expression in the square brackets of Equation 14 is of the same order, that is 10^{10} . Assuming the radius of the drop to be $r_0 \sim 10^{-3}$ cm., we find $V \sim 10^{-8}$ cm.³ Taking $M_c \sim 100$, and neglecting the surface force, we find that

$$f \sim \text{grad } c. \quad (23)$$

As to the possible values of $\text{grad } c$, we can get an idea of them from data published by J. Weichherz, on glucose fermentation. From Fig. 5 of (30) we see that $q \sim -10^{-2} \frac{gr}{\text{min.}}$ cm.³ On page 344 of (30) we find for

$$D_c = 2.8 \times 10^{-4} \frac{\text{cm.}^2}{\text{min.}}$$

Hence, using our equation (21), we find

$$c \sim c_0 - \frac{10^{-3}}{r},$$

or

$$\frac{dc}{dr} \sim \frac{10^{-3}}{r^2}.$$

For distances comparable with the radius of the drop, that is $r \sim 10^{-3}$ cm., we find $\frac{dc}{dr} \sim 10^{-2} \frac{gr}{\text{cm.}^4}$ and hence, with regard to equation (23),

$$f \sim 10^{-2} \text{ dyn.}$$

Per cm.³ this gives a tremendous force of about 1 kg.

Assuming the viscosity η of the surrounding medium to be that of water, we find by using Stokes' formula (5, p. 85)

$$u = \frac{f}{6\pi\eta r_0}$$

that under the influence of such a force, if the latter would be uniform over a comparatively large region, the drop would acquire a velocity of

$$u = 50 \frac{\text{cm.}}{\text{sec.}}$$

By taking motion pictures of suspensions of cells which exert on each other any kind of forces, the velocity with which they approach each other, or move away from each other, could be measured, and applying Stokes' formula in case of spherical cells the forces could be estimated. This perhaps is a way to a quantitative study of such phenomena.

It is important to discuss how such forces would compare with electrical forces, which play a prominent part in the living cell. Although the actual potentials are only of the order of millivolts, the voltage gradients may be extremely high, due to the very small thickness of the electrical double layers, which are the seats of bioelectric phenomena (3). But just this very small thickness of these double layers (10^{-6} cm.) causes the range of the strong electrical forces to be restricted to very small distances. In order to estimate the force which may act on a unit whose size is comparable with that of a cell, we must consider voltage-gradients on a correspondingly larger scale. But if two parts of a cell or a tissue, distant 10^{-3} cm., have a difference of potential of even 100 mv. this gives a voltage gradient of $\sim 3 \times 10^{-1}$ cgs. The surface density of the electric charge of a colloidal particle for potentials of the order of 50 millivolts is $\sim 10^3$ electrostatic unit cgs. (5, Chapter 11), which gives for the total charge of a particle of the radius $r_0 \sim 10^{-3}$ cm. the value $\sim 10^{-2}$ electrostatic unit cgs. The force acting on such a particle in a field of the above voltage gradient will be only $\sim 3 \times 10^{-3}$ dyn. That is, the forces here considered may exceed considerably the electrical forces. Of course, in other cases, like those of electric organs, the latter may be much higher than estimated here.

As regards the variation of the force with distance, this may be of various kinds. If in equations (14) and (16) the last member in the square brackets is very small as compared with the others, which often may be the case, and if

$$\frac{d\gamma(c)}{dc} = \text{const.},$$

then the force becomes proportional to *grad c*. If *c* is given by equation (21), then *f* varies as the inverse square of the distance.

However, in general the law of variation with distance will be much more complicated. Especially the force due to the variation of surface tension may vary in a somewhat unusual way. P. Lecomte du Noüy (15) found, that for many capillary active substances the surface tension of the solvent decreases with increasing concentration of the solution, reaches a minimum and then again increases. If the

concentration *c* decreases with distance as $\frac{1}{r}$ (Equation 21), there will

be a certain distance *r*₀, for which *c* has just the critical value, corresponding to the minimum surface tension of A. It is easily seen, that

for $r > r_0$, $\frac{d\gamma}{dc} < 0$, and for $r < r_0$, $\frac{d\gamma}{dc} > 0$. In other words, the force

will be an attraction for $r > r_0$, and a repulsion for $r < r_0$.

II

Let us now discuss briefly what phenomena will result from the existence of the above forces.

First of all, it follows from the foregoing that not only whole cells will exert on each other forces of attraction and repulsion, but also the various parts or constituents of a single cell will do this. Since not only these different constituents possess a mobility in the cell, but also cells as a whole are sometimes loosely enough connected with each other so as to allow relative displacements and migration of the cells (14, p. 210-20, 25), the geometrical arrangement of the various cells with respect to each other as well as the internal arrangement of the inner constituents of the cell will be determined by the equilibrium of those forces. In the arrangement of the constituents of the individual cells, the electric forces, as said above, may be just as important.

From equation (8) it follows that those forces have a potential and therefore the problem of the configuration of a group of cells in a tissue reduces to a problem of mechanical equilibrium. It is true, that as long as we do not know the nature of all the substances which

participate in generating these forces, and hence as long as we do not know the actual distribution and magnitudes of the forces, practically nothing is gained by this reduction to a mechanical equilibrium problem. At the present stage of the problem it would be far too optimistic to attempt an explanation of the phenomena of determination of organic form. The logical way to be followed is to investigate mathematically the various simplest possible forms, which may be obtained under various assumptions about the substances, which cause these forces. This will be reserved for a future publication.

Here let us indicate one important consequence from the foregoing:

Imagine an aggregation of growing and dividing cells, distributed at random, in which their constituents are also distributed at random. Under such conditions the planes of division of the individual cells will also be distributed at random, and as a result of this, the growth of all elements of volume proceeding with equal probability in all directions, the aggregation will remain a shapeless amorphous mass. But all this holds true only if the above discussed forces are either absent, or negligible. If such is not the case, not only will the individual cells tend to arrange themselves into definite geometrical pattern under the influence of the forces, but in general the internal constituents of the heterogeneous cells will also be redistributed according to a definite law. Inasmuch as the distribution of various materials within the cell influences the direction of the plane of division, these planes in various cells of the aggregate will no longer be distributed entirely at random. This does not mean necessarily that they will all be parallel. Such could be the case only under rather special conditions. But there will be a certain preferred direction of division, and in general this preferred direction may be different in different parts of the now geometrically more definite aggregation. As a result of this, the aggregation will grow at different rates in different directions and will become an anisotropic tissue, endowed with definite form.

Now let us again consider the shapeless aggregations, supposing that due to the particular nature of the substances produced by the cells, the cytotropic forces are so small that they remain without any effect on the geometrical arrangement of the cells and of their constituents.

Let us implant into this aggregation a group of other cells of a different nature, which produce substances acting very strongly cytotropically. These substances will diffuse into the shapeless aggregation, and thus a definite field of force will be created around the implanted group of cells. It must be emphasized that this field of force will not necessarily be symmetrical with respect to the implanted group. If, due to the mutual interaction of the implanted cells, the implanted group possesses already a definite anisotropy or structure, and if the substances in question are produced only in definite parts of the cells, the geometrical configuration of the field of force around the transplanted group may be a very complicated, highly asymmetrical one. But the field will be completely determined by the character of the structure of the implanted group. Under the influence of this field of force, the cells of the shapeless aggregate which are adjacent to the implanted part will undergo a rearrangement, and instead of growing at the same rate in all directions, the parts of the aggregate adjacent to the implanted part will acquire an anisotropy of growth. Whereas in the absence of the implanted part, the aggregate would have grown into an amorphous, shapeless mass, it will grow now into a definite tissue, the structure of which will be determined by the field of force, around the implanted part, or in other words, by the nature of the implanted part. Similarly, we may expect an "organizing" effect on a non-organized group of cells transplanted into an organized tissue. Such phenomena, known as "organization" and "induction," are actually observed in embryological development (1, 12, 13, 26-29).

SUMMARY

From thermodynamical considerations expressions are derived for the forces which will act on a liquid drop suspended in another liquid, if the latter contains in solution a substance whose concentration is non-uniform. It is shown that forces of attraction and repulsion between two such drops may result, if the drops are seats of chemical reactions, which produce substances diffusing into the surrounding medium.

An estimate of the order of magnitude of such forces is made, and it is found that they may exceed the electrical forces which would result from the charges which the drops may possess.

A possible experimental method of studying such forces is suggested.

The possible connection of the above forces with those observed in the phenomena of "cytotropism," and their bearing on the physical explanation of organic form, especially of the phenomena of "induction," is briefly discussed.

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THE SENSIBILITY OF THE SUN-FISH, *LEPOMIS*, TO MONO-CHROMATIC RADIATION OF LOW INTENSITIES

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INTRODUCTION

Measurements on the sensibility of *Lepomis* to very dim spectral lights have been made with the purpose of testing the hypothesis that the photosensitive retinal pigment, known as visual purple, is responsible for vision at low intensities of illumination.

Since the pioneer morphological investigation of Schultze (1866), it has been known that the vertebrate eye usually contains two kinds of photoreceptors. In man, this duality of anatomical structure is paralleled physiologically by the presence of two, qualitatively different, kinds of vision. Visual sensation produced by high intensities is accompanied by color perception, whereas visual sensation produced by very low intensities—even of spectral lights—is indistinguishable from that caused by white light.

These dim spectral lights are not, however, identical in their efficiency. Less energy of the wave-length $500\text{ m}\mu$ is needed to produce a sensation of given brightness than is necessary if lights of the wave-lengths $450\text{ m}\mu$ or $600\text{ m}\mu$ are used, although these three spectral lights cannot be told apart as to their colors. The quantitative description of these differences in the effectiveness of various spectral

* It is a pleasure to express my gratitude to Professor Selig Hecht, who suggested this problem, for his constant help and encouragement during its progress. The members of the Laboratory of Biophysics have aided very generously in every possible way. This work was done during tenure of the University and Gottsberger Memorial Fellowships, for which I am indebted to the Trustees of Columbia University. Preliminary experiments and the absorption curve measurements were carried out at Woods Hole during the summers of 1927-29. I wish to thank the Director of the Marine Biological Laboratory for the facilities placed at my disposal.

lights for liminal perception by the human eye is called the scotopic (or dim vision) visibility curve. It was first obtained by Hering and Hillebrand (Hillebrand, 1889) and is shown as the continuous curve of Fig. 1, taken from the more recent and accurate determinations of Hecht and Williams (1922). According to the Duplicity Theory

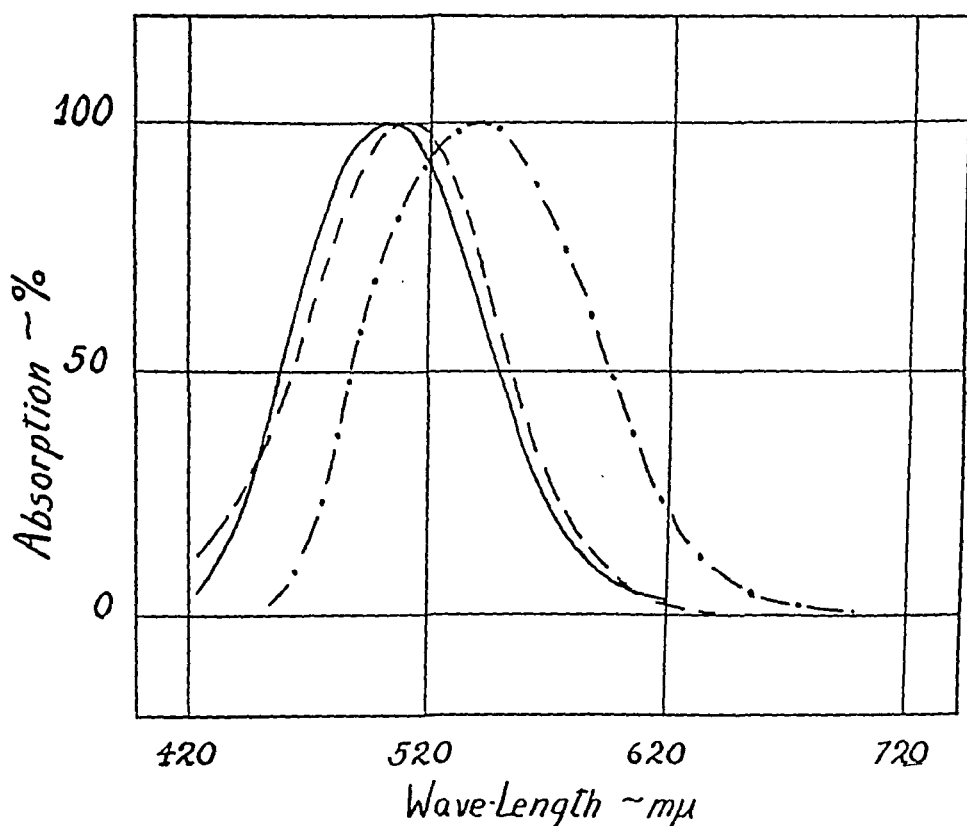


FIG. 1. Visual purple and vision. The continuous line is the absorption spectrum of frog visual purple (Koettgen and Abelsdorff); the broken line is the visibility curve of the human eye at low illumination (Hecht and Williams); and the dot and dash line is the absorption spectrum of fish visual purple (Koettgen and Abelsdorff).

proposed by von Kries in 1894, the rods are the functional elements responsible for dim vision.

Boll (1876) discovered that there is in the rods a sensitive pigment which has since come to be known as visual purple. This substance, which has a reddish color, has been further investigated by Kühne

and his coworkers (Ewald and Kühne, 1878) who found that it can be extracted from the retina with a solution of bile salts. It is bleached by light both in the retina and in solution. *In situ*, it regenerates in the absence of light. These two important properties immediately led to the assumption that visual purple is the photosensory substance responsible for vision. This was especially so since Kühne pointed out that the effectiveness of spectral lights in bleaching visual purple is roughly proportional to their effectiveness in vision.

However, visual purple has never been found in the cones, which, in man, are by far the most important visual sensory elements. On the other hand, Koenig (1894) and Trendelenburg (1905) have brought forward evidence for the hypothesis that visual purple is responsible for dim vision. It is from this restricted hypothesis that the theoretical significance of the scotopic visibility curve is derived. The evidence in favor of this hypothesis depends upon the visibility curve, and is two fold.

First, Koenig and others have shown that the scotopic visibility function is practically identical in form with the absorption spectrum of visual purple. That is, visual purple absorbs the various spectral lights in the same proportions as they are effective for the eye (Koenig, 1894; Koettgen and Abelsdorff, 1896). Second, the curve describing the rate of bleaching of visual purple by spectral lights is also very similar to the dim-visibility curve. In other words, the relative effectiveness of spectral lights in bleaching visual purple is similar to their relative capacity for stimulating the eye (Trendelenburg, 1905).

This similarity between the bleaching curve of visual purple, its absorption spectrum, and the human dim-visibility curve is very striking. A direct relation between the first two follows, of course, from the Grotthus-Draper law of photochemistry. Bleaching is the effect of light on visual purple and this law states that lights are effective only to the extents that they are absorbed. The close correlations between these two curves and the visibility function, unless purely fortuitous, follows, however, only if bleaching of visual purple by light is the primary cause of dim vision.

The hypothesis that visual purple is responsible for dim vision rests, therefore, mainly on the experimental fact that the human scotopic visibility curve is similar in form to the absorption spectrum

of human visual purple. The possibility that this agreement may be fortuitous is not entirely removed by the fact that it is found in some other vertebrates as well, since all the animals studied so far (frog, cat, and fowl) have similar visual purples and show similar visibility curves.

A further test of this hypothesis can, however, be made. Koettgen and Abelsdorff examined thoroughly the absorption spectra of visual purple extracts from all the vertebrate classes in which this pigment can be obtained. They found that visual purples from mammals, birds, and amphibians all give one type of absorption spectrum which is identical with that found by Koenig for human visual purple. This absorption spectrum is shown in Fig. 1 in broken line. There is a systematic difference between the absorption and visibility curves, the latter being shifted 7-8 $m\mu$ towards the red end of the spectrum. This difference has been interpreted (Hecht and Williams, 1922) on the basis of Kundt's rule, as due to the difference in the refractive index of the solvent media in the two cases. The outer portions of the rods, where visual purple is found, are highly refractive bodies and Kundt's rule states that when a substance is dissolved in two different media its absorption spectrum in the solvent of higher refractive index is shifted to the red.

In Fig. 1 is also shown the absorption spectrum of the visual purple found by Koettgen and Abelsdorff in eleven species of fish. It has essentially the same shape as the other curve but is shifted to the red so that its maximum is near 540 $m\mu$ instead of 500 $m\mu$. Because fish visual purple is different from that of all other vertebrates, it is possible to test the hypothesis that visual purple determines the visibility function at low intensities, since, according to this hypothesis, the visibility curve of fish when measured at low intensities should be different from that of other vertebrates and should be determined by the absorption spectrum of the corresponding visual purple.

There have been few attempts to obtain the visibility function of vertebrates other than man. Chaffee and Hampson (1924) have presented very good data on the excised frog retina. Honigmann (1921) and Murr (1928) measured the visibility curves of the living fowl and cat, respectively. The only measurements on fish revealed in the literature have been made by von Hess (1909). He placed a school

of young, positively phototropic fish in a glass tank one side of which was illuminated by two adjoining patches of light. One patch was produced by a source of variable spectral composition. The other was formed by a white light whose intensity could be varied. By adjusting the brightness of the white patch until 50 per cent of the fish collected at either field, Hess measured the amount of white light necessary to balance a number of spectral lights. In his paper, he gives a plot of these "white valences" against the wave-lengths of the spectral lights and concludes that it is similar to Hering's plot of the scotopic visibility curves of normal and color-blind men. A close examination of Hess' evidence reveals, however, many weaknesses. He gives no numerical data, makes no correction for the energy distribution in the spectrum of his source of light, and fails to show any data at the critical points which would determine the curve.

It has therefore been necessary to investigate the visibility function of fish at very low spectral illuminations, so as to secure measurements capable of testing the hypothesis that visual purple determines this function in dim vision.

The Method and Apparatus Used

The visibility curve of fish, determined for the purpose just described, must ultimately be compared with that of man because the latter has been obtained very accurately by numerous observers, and constitutes, in a sense, a standard of comparison for measurements on other vertebrates. Accordingly, it was decided to obtain the data on living, individual fish by a method which would not depend on any previous training or conditioning of the animal. Measurements obtained under these restrictions are strictly comparable to those on the human eye.

Of the several methods which have been employed in previous work, only that of Honigsmann satisfies the conditions imposed. The method used by Chaffee and Hampson for the frog depends on the measurement of the energy of various spectral lights which is necessary to produce a constant action potential. However, it was used only on excised eyes. The Dressur technique, which is well known through the work of von Frisch and his students, has achieved many triumphs but, since its essence involves training, it runs counter to our restrictions. Finally, there is the method used by Hess that has already been described, which is statistical in nature, depending on the average response of a large group of animals.

These self-imposed restrictions have necessitated the development of a method which determines whether the individual animal can see a light of given energy content. This method takes advantage of the well known fact that a fish follows,

with its body, a movement in its visual field. This response to a movement is found in many other animals and belongs to the general class of tropisms. Rather unfortunately, it is known as the "rheotropic reaction," a misnomer antedating Lyon's proof in 1904 that it is a response to a visual stimulus and not to a water current. Main (1927) has investigated this reaction in *Fundulus*, and a similar response has also been used recently in the determination of the visual acuity of

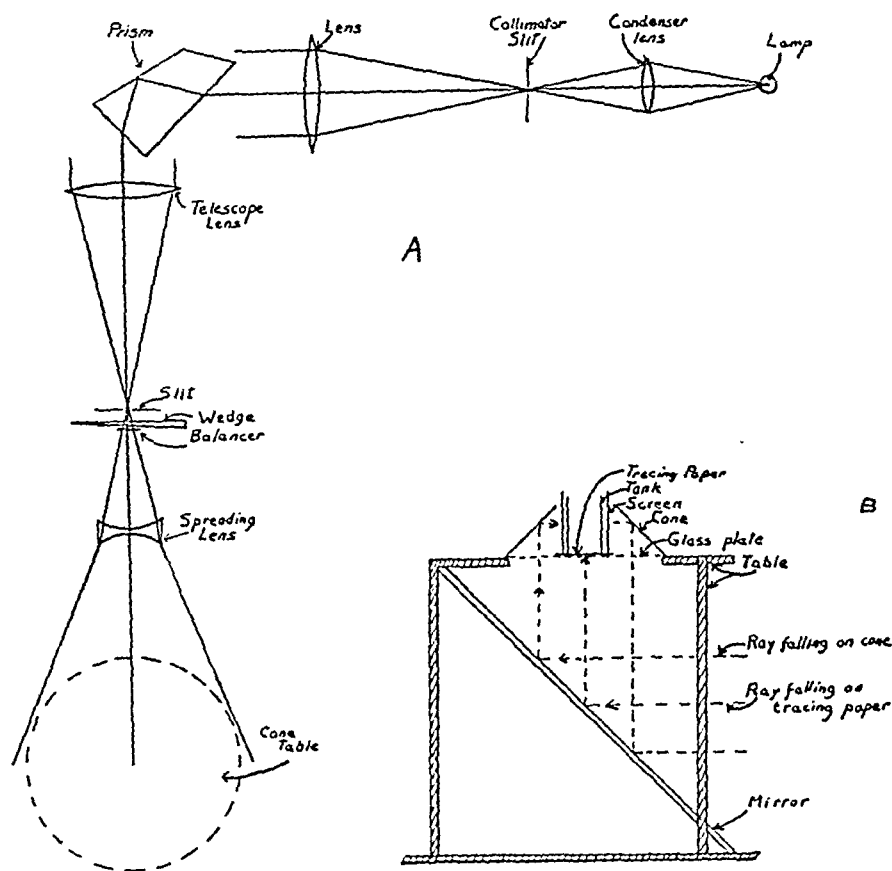


FIG. 2. Arrangement of apparatus. A is a top view showing diagrammatically the disposition of the set-up. B is a vertical section of the box holding the mirror, screen, tank, and animal.

the honey bee (Hecht and Wolf, 1929) and of *Drosophila* (Hecht, Wolf, and Wald, 1929).

The common sun-fish, *Lepomis*, is particularly accurate in its response to a moving visual stimulus and has therefore been employed in these experiments. It is placed, (Fig. 2), in a cylindrical glass jar on a glass-topped table. Around the outside of this tank is a cylindrical screen composed of equal and alternate vertical

bars and spaces. These bars are made by milling out alternate 2 mm. strips from a thin brass sheet 32 cm. long and 15 cm. wide, almost to the long edge and parallel to the width, and then joining the edges to form a hollow cylinder. A beam of light, sent up through the glass table top, is reflected through this screen and towards the animal in the tank by a hollow, truncated 45° cone made of plaster of Paris. The bottom of the glass jar is covered on the outside with translucent paper so that the portion of the light beam which falls on it directly produces an even and dimly illuminated background against which the animal can be observed.

When the illumination is sufficiently bright, rotation of the screen at the rate of 1 cm. per second causes the fish, which is generally quite motionless, to swim suddenly in the direction of the rotation. Upon reversing the motion of the screen, the fish reverses its direction. The animal responds in this way to continued reversals of the screen for minutes at a time.

On decreasing the illumination, a point is reached at which no response is produced. The minimum intensity at which the fish still responds varies with the size of the bars and spaces which make up the screen. The intensity or, more precisely, the energy content of the beam at which the reaction is just elicited varies also with the wave-length of the light. Thus, by using one constant screen and varying the spectral composition of the illumination, it is possible to obtain the visibility curve of a single individual. The experimental procedure is a matter of determining the minimal amounts of various spectral energies that are needed to produce a response by the fish to a rotation of the screen.

As already mentioned, the screen is composed of elements which are 2 mm. wide. Since its distance from the animal's eye is approximately 1 cm., the corresponding visual acuity is about 0.01 Snellen units. It was first shown accurately by Koenig that the human eye needs very low illumination indeed, to possess this visual acuity. *Lepomis*, also, requires very dim illuminations to respond to the rotation of the screen. The brightness at which these measurements have been made is of the order of 10^{-6} millilamberts. These measurements accordingly describe the relative efficiency of spectral lights for the dim vision of the sun-fish.

This method, in general, assumes that the visibility curve obtained by measuring the relative effectiveness of various spectral lights in permitting the discrimination of a constant pattern are comparable to the curve determined for a constant level of brightness sensation. I have tested this assumption by using this method to measure my own visibility curve. A single set of determinations was sufficient to show that the curve thus obtained is identical with that obtained by other methods. These data are reproduced in Fig. 3 together with the curve of Hecht and Williams which is an average of many determinations on different individuals.

Spectral lights are obtained in these experiments from a Hilger constant deviation spectrometer having a symmetrical front slit. Both this slit and that on the collimator are open only 0.5 mm., so that fairly homogeneous spectral bands are used. The purity of the band varies, of course, with the wave-length, but its width is not greater than 2 μ . The light source is an 18a. 6v. Mazda lamp with a vertical ribbon filament.

The energy content of the beam is measured by means of a Hilger thermopile and a Leeds and Northrup type HS galvanometer. In the earlier experiments, Corning glass filters were used to reduce stray light. Their spectral transmissions have been measured spectrophotometrically, and the proper corrections made in the data.

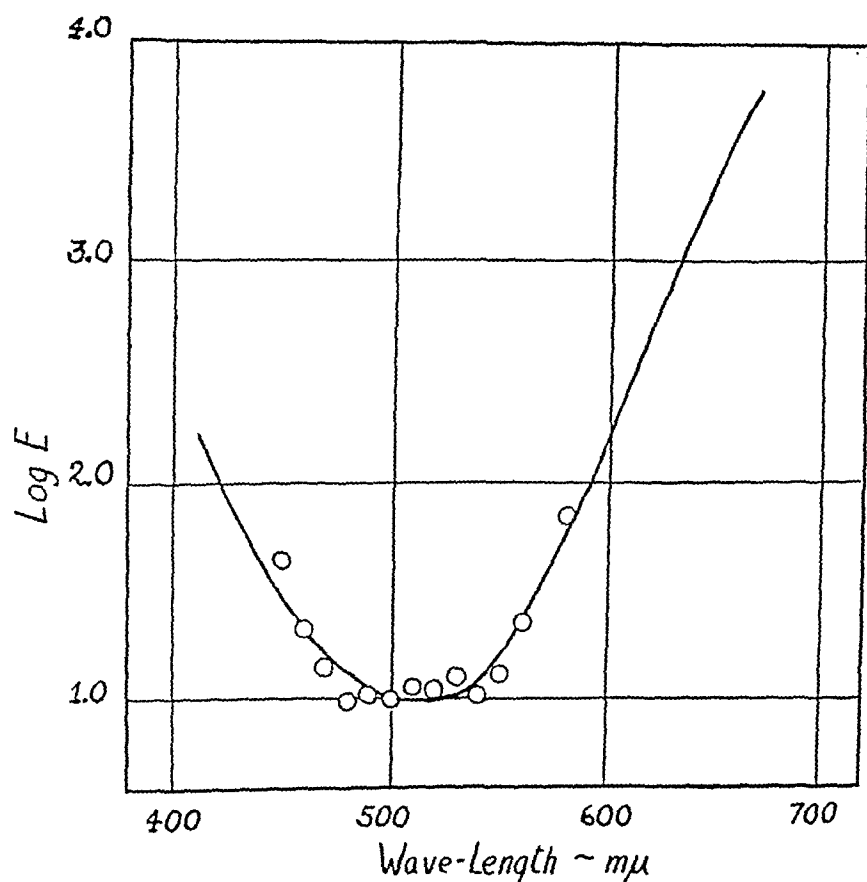


FIG. 3. Human visibility function at low illumination. The points were determined for the eyes of H. G. by the moving stripe method described in the text; the smooth curve is that obtained by Hecht and Williams with 48 observers using the equality of brightness method. The two methods apparently give similar results.

The intensity is varied by passing the beam through any desired portion of an Eastman Kodak balanced neutral wedge which is mounted in a movable carriage designed by Mr. Simon Shlaer of this laboratory. The wedge is calibrated photometrically so that the transmission at any given setting of a scale which is mounted with it is accurately known. The available portion of the wedge is 13 cm. long and

permits a gradual change in the intensity over a range of approximately 1:1,000. The position can be read accurately to 0.1 mm., although the measurements during these experiments have rarely been to closer than 1 mm. The transmission of the wedge is described by the equation

$$\text{Log } I = 3.308 - 0.208x \quad (1)$$

where x is the scale reading in cm. and $\log I$ is expressed in arbitrary units. Since the dye mixture used in the wedge is not quite neutral, spectrophotometric calibration of its spectral transmission has been made and proper corrections have been applied. In certain cases, Wratten neutral filters have been used to supplement the wedge. These have also been calibrated spectrophotometrically, and the data corrected accordingly. Special attention is called to these corrections because they are usually ignored on the supposition that such wedges and filters are really neutral; their neglect here would produce distinct errors.

Procedure

Thirteen animals have been used in these experiments. The first three were obtained from a dealer while the others were supplied by the New York Aquarium, through the courtesy of Mr. Breder. Only those animals which responded best to a moving screen have been used. Little difficulty has been found in keeping the fish in the laboratory for about 6 months, each individual living in a separate balanced tank.

A standardized experimental procedure has been adopted in this work. The cylindrical jar is cleaned before use and filled with 250 cc. of filtered tap water. Since it is about 9 cm. inside diameter, the depth of water is 4 cm. The animal to be used is then transferred from its home tank into the experimental one and is placed in the apparatus. It is allowed to become dark adapted for at least 2 hours. 15 minutes before the end of this adaptation period, the observer enters the dark room to become dark adapted also. During this time the spectroscope is set and the lamp current adjusted to the correct amperage.

When the observer has become sufficiently dark adapted, the shield around the cone table is removed, the wedge set at its densest point, and the shutter on the spectroscope then opened. The screen is rotated in either direction. If the animal does not respond, the wedge is moved out about 5 mm. to increase the intensity of the light and the screen is again rotated. This procedure is repeated until the animal gives a definite reaction. The light is again dimmed by moving the wedge in several mm. By gradual search over these last few divisions, a setting of the wedge is obtained at which the animal still responds, whereas it does not do so at the next small decrease of the intensity. The scale reading is recorded, and the measurement is repeated. The spectroscope is then set for another wavelength, and a measurement made. The order of the readings is purposely made irregular. The time elapsed in a complete experiment is about 2 hours, exclusive of the adaptation period.

It has been possible to determine the minimum energy required by fish to respond to the moving screen for wave-lengths between 450 $m\mu$ and 600 $m\mu$. Determinations could not be made at shorter wave-lengths because the energy emitted by the source in that region is not great enough. The long wave limit is determined by the fact that the animals seemed to be more sensitive to these wave-lengths than the observer, and appeared to respond to intensities below those needed to give sufficient illumination on the background for accurate observation.

TABLE I

Example of calculations of relative effectiveness of different portions of the spectrum. Animal 6AII. The combined correction factor includes correction for the energy distribution in the spectrum and for the spectral transmissions of the wedge and filters. $\log E_{\lambda\max}$ is 2.090.

λ	Wedge reading	$\log I$	\log combined correction factor	$\log E_{\lambda}$	Relative effectiveness $\frac{E_{\lambda\max}}{E_{\lambda}}$
480	7.9	1.660	1.129	2.789	20.0
500	9.7	1.285	1.272	2.557	34.1
510	10.7	1.075	1.330	2.405	48.4
520	11.2	0.975	1.411	2.386	50.6
530	12.6	0.680	1.504	2.184	80.5
540	13.4	0.515	1.597	2.107	96.2
550	12.9	0.620	1.683	2.303	61.2
560	12.4	0.720	1.763	2.483	40.5
580	11.2	0.975	1.862	2.837	17.9
600	9.9	1.245	1.920	3.165	8.4

The measurements for any given wave-length are obtained in terms of the wedge scale reading. By means of Equation 1, these values are converted into the logarithms of an arbitrary intensity scale. Since the visibility function is best expressed in per cent of the most efficient wave-length, this arbitrary scale is entirely adequate. One absolute value has already been mentioned. At 540 $m\mu$, with a wedge setting of 14.0 cm. corresponding in order of magnitude to the intensities used, the reflecting face of the plaster of Paris cone has a brightness of 1×10^{-6} millilamberts.

In calculating the minimum energy at any wave-length necessary

to cause an oriented response by the sun-fish, there are added to the log I values obtained from the data the logarithms of the various correction factors which have already been described. The use of these correction factors eliminates from the measurements the differences

TABLE II
Relative Effectiveness of Spectral Radiation for Lepomis

Animal	Wave-Length λ										
	480	500	510	520	530	540	550	560	570	580	600
1		12.4	18.9	19.8	30.6	90.5	58.6	47.7	8.4		
2		14.6	17.4	16.6	42.3	97.3	59.0	44.5	9.0		
3		10.7	17.8	13.8	51.4	93.6	83.4	50.7	4.7		
1BI	18.5	19.0	27.6	24.6	28.9	89.3	53.8	10.5	9.7	1.3	1.2
1BII	48.8	37.6	54.0		42.8	91.6	48.6	27.1			
2AI	17.7	22.0	24.0	22.7	35.5	95.7	44.7	9.2	11.2	2.1	0.9
2AII	18.7	26.1	29.2	28.1	75.2	77.3	49.8	26.7	28.4	21.0	7.1
3AI		6.8	9.8			91.4	20.9	7.6	4.4	1.9	
3AII	15.1	17.4			40.7	98.4	37.1	11.5		6.1	
4AI		16.7	27.6			94.6	46.4	16.7		2.8	1.0
4AII			28.9	81.5	63.3	98.0	56.4	18.3	10.9	4.3	2.3
4AIII	10.5	18.3			29.6	91.4	29.6	7.6		5.3	
4AIV	9.4	10.6	15.4	11.9	12.3	96.4	26.4	13.3		7.6	
4AV		20.3		29.7	36.2	91.6	42.9			19.2	3.6
4AVI	10.5	26.3	19.5	19.5	90.4	80.0	68.7			6.9	4.8
5AII			23.2	63.2	57.7	89.3	45.8	16.1	14.5	3.3	2.8
5AIII	27.0	47.7	56.2	68.4	78.7	96.2	65.6	26.4		19.2	
6AI		8.3	18.2			91.4	23.5	8.3	3.8	2.0	
6AII	20.0	34.1	48.4	50.6	80.5	96.2	61.2	40.5		17.9	8.4
8III	51.4	51.1	49.9	47.8	53.5	94.0	76.6	43.4	34.2	22.8	8.8
9	19.1	32.2	37.2	41.2	57.0	92.9	65.6	50.9	20.4	11.4	4.9
1NS	48.2	43.3	49.9	35.0	47.3	82.8	82.8	61.0	41.9	8.5	
2NS	38.6	43.9	31.1	29.5	45.9	96.6	63.5	42.8	19.7	19.9	17.8
Average...	25.25	24.73	30.21	33.55	52.62	92.02	52.65	27.66	15.80	9.66	5.30
8I	42.8	78.3	77.6	89.9	86.3	95.3	92.0	59.8	43.1	19.4	12.3
8II		68.1		77.5	73.5	80.4	98.6	55.9		16.9	

due to the apparatus; the values then refer only to the relative effectiveness with which the various wave-lengths stimulate the eye.

These corrected values, which are now in terms of the logarithms of the relative energy, are plotted against the wave-lengths for which they have been obtained. A smooth curve is drawn through the points

to find the most effective wave-length. The reciprocal of the minimum energy ($E\lambda$) at any wave-length (λ), divided by the reciprocal of the minimum energy ($E\lambda_{\max}$) required at the most effective wave-length (λ_{\max}) gives the efficiency of the wave-length (λ) in per cent. An example of the steps in the calculations of a single series of measurements is given in Table I.

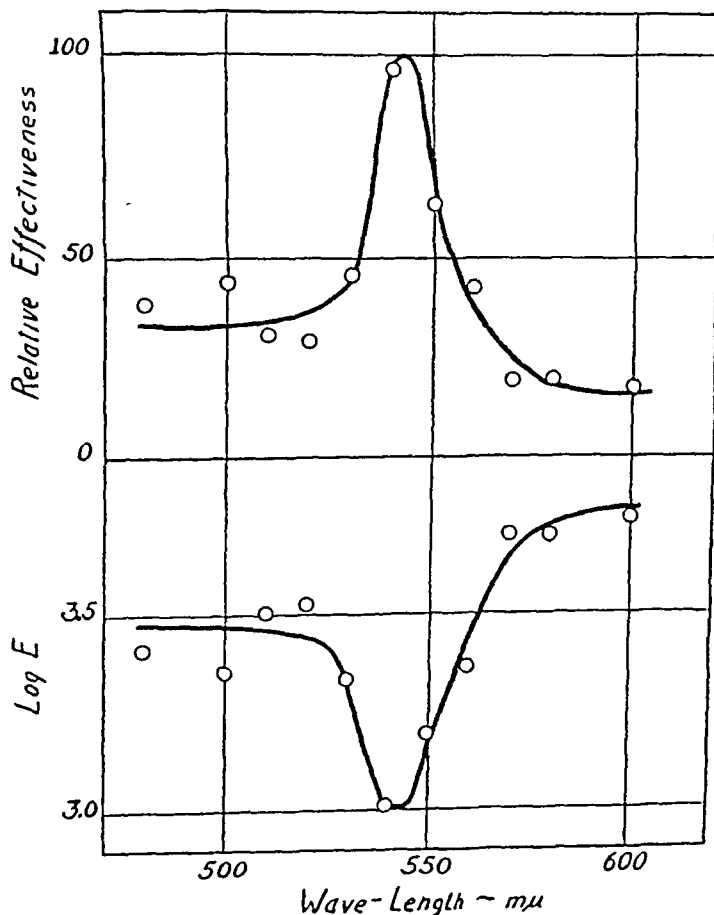


FIG. 4. Spectral visibility function of *Lepomis* at low illuminations. The data are for Animal 2NS and are typical. Compare this with the average in Fig. 5.

Data

Twenty-five sets of data have been obtained on the thirteen animals used. They are presented in Table II. Of the twenty-five sets, twenty-three have been averaged and their average is included in

Table II. The two runs 8I and 8II have been tabulated separately; the results with this Animal 8 are exceptional. The significance of these exceptional results will be discussed in a following section.

In Fig. 4 there is shown the result obtained with a single animal. It is entirely typical. The averaged measurements for the twenty-three series are shown in Fig. 5. It is apparent from Table II and Figs. 4 and 5, that the most effective wave-length, in every case, lies

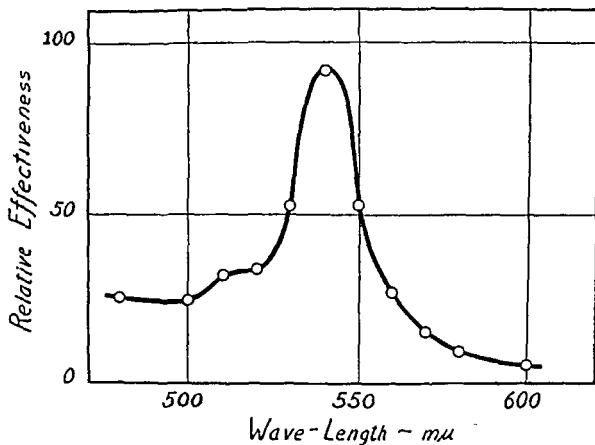


FIG. 5. Spectral visibility function of *Lepomis* at low illuminations. The points are the average of twenty-three series of measurements with thirteen animals.

between 535–545 μ . The efficiency drops very rapidly on the long wave side of the maximum until at 600 μ it is only about 10 per cent. On the short wave side, the drop is equally steep but stops at 520–530 μ . From there on, the curve is either flat or there may be a slight hump. This portion of the curve is variable among the different animals and even from day to day in the same individual.

The Absorption Spectrum of Lepomis Visual Purple

Comparison of the absorption spectrum of fish visual purple, taken from the data of Koettgen and Abelsdorff (shown in Fig. 1) with the visibility curves given in Figs. 4 and 5, reveals that while both maxima are approximately at the same wave-length, the shapes of the two curves are entirely different. If visual purple determines the dim-visibility function, both the shapes and the maxima of the two curves should be almost identical. There is, however, the possibility that *Lepomis* visual purple is different from that of the eleven species of fish examined by Koettgen and Abelsdorff. This is ruled out by the measurements of the absorption spectrum of *Lepomis* visual purple which I carried out during the summer of 1929 at Woods Hole.

Extracts of visual purple have been made from the eyes of *Lepomis*, in a solution of bile salts, after the method developed by Kühne. The modification described by Hecht (1920) has been used. Since it was found difficult to remove the retina from the optic cup, the enucleated eye has been used for the extraction in most cases.

The procedure is as follows. About twenty sun-fish, all approximately 15 cm. long, are dark adapted in the dark room aquarium for a period ranging from 4 hours to overnight. They are taken out in a dim red light, and their heads are chopped off immediately, using a heavy fish knife pivoted at one end. As the head is severed, it is thrown into a large tank containing 10 liters of Ringer's solution. This tank is shielded from even the faint red light; the large volume of water it contains promotes removal of blood from the head.

When all the heads have been collected, the eyes are dissected out by making three incisions in the head, one through the top and one through each cheek. The eyes are then raised out with a curved forceps, and the cornea cut off with a fine scissors. The lens and humor are squeezed out gently and the enucleated eye is pulled out of the head and dropped into a jar of fresh Ringer's solution. When all the eyes are thus removed, the liquid is changed; and the eyes are then placed in centrifuge tubes and covered with more Ringer's solution.

After centrifuging in the dark for about fifteen minutes at a speed of 2300 R.P.M., the supernatant liquid, containing substances soluble in Ringer's solution, is poured off. 10 cc. of an aqueous 4 per cent bile salts solution are added. The eyes are shaken up in it, and the mixture allowed to stand in the dark for 30 minutes. On centrifuging again, a reddish solution is obtained which contains the visual purple. It is used as fresh as possible and kept at 0°C. when not in use.

The bile salts were prepared from the best obtainable commercial grade by decolorizing with charcoal in an alcoholic solution until only a faint trace remained of the yellow pigment present in the bought product.

The solution of visual purple obtained as described, is too concentrated for accurate absorption measurements and dilution to a convenient concentration must be made. The absorption measurements have been made with a single chambered cell, presenting a layer of solution 5 mm. deep, and a Koenig-Martens spectrophotometer. The cell is held in a special fitting on the stage of the instrument to eliminate any movement.

In order to decrease the bleaching effect of the measuring light, Corning colored filters have been used to eliminate lights of unnecessary wave-lengths. The intensity of the source is also cut down by means of a rheostat to the lowest point

TABLE III
Absorption Spectrum of Lepomis Visual Purple

λ	Absorption in per cent					
	July 17	July 18	Aug. 23A	Aug. 23B	Aug. 28	Sept. 5
460	51.6					
470		47.8				
480	72.6	53.2				44.7
500	77.4	79.0	53.8	52.7	91.1	73.9
510	78.9					
520	87.9	90.7	70.2	84.1	98.9	84.7
530	98.4	98.0		87.1	99.3	98.7
540	100.0	86.3	97.6	94.0	87.9	96.3
550	99.5	94.1	91.7	97.6	80.3	80.7
560		83.9	72.0	76.6	85.1	72.1
570	67.4					
580	75.3	78.0	55.5	64.0	58.3	48.9
590	53.2					
600	47.4	60.5	45.0	53.1	30.8	23.2
620	15.3	52.2		38.7	17.8	7.1

at which readings can be made without loss of accuracy. The readings have been scattered and also repeated in the reverse order to compensate for possible bleaching. When the measurements are finished, the visual purple is bleached without moving the cell by exposure to a 50 watt lamp for 20 minutes, and the absorption curve of the bleached substance is then determined. The difference between the two absorption curves represents the absorption spectrum of visual purple.

Difficulty has been found in obtaining consistent results. This difficulty is not due to technical errors since similar measurements on frog visual purple check very well with those of Koettgen and Abelsdorff. The visual purple of *Lepomis* often becomes cloudy in a very short while and also bleaches rather more quickly than does that of the frog. These two factors are probably responsible for the difficulty.

Eight sets of data (shown in Table III and in Fig. 6) have been obtained by using fresh solutions and taking a few rapid readings at well scattered points. These are in general agreement with the measurements of Koettgen and Abelsdorff. Whatever may be the explanation for the greater variability in the data on the absorption spectrum of *Lepomis* visual purple, the averaged curve has the same maximum as that obtained for other fish by Koettgen and Abelsdorff.

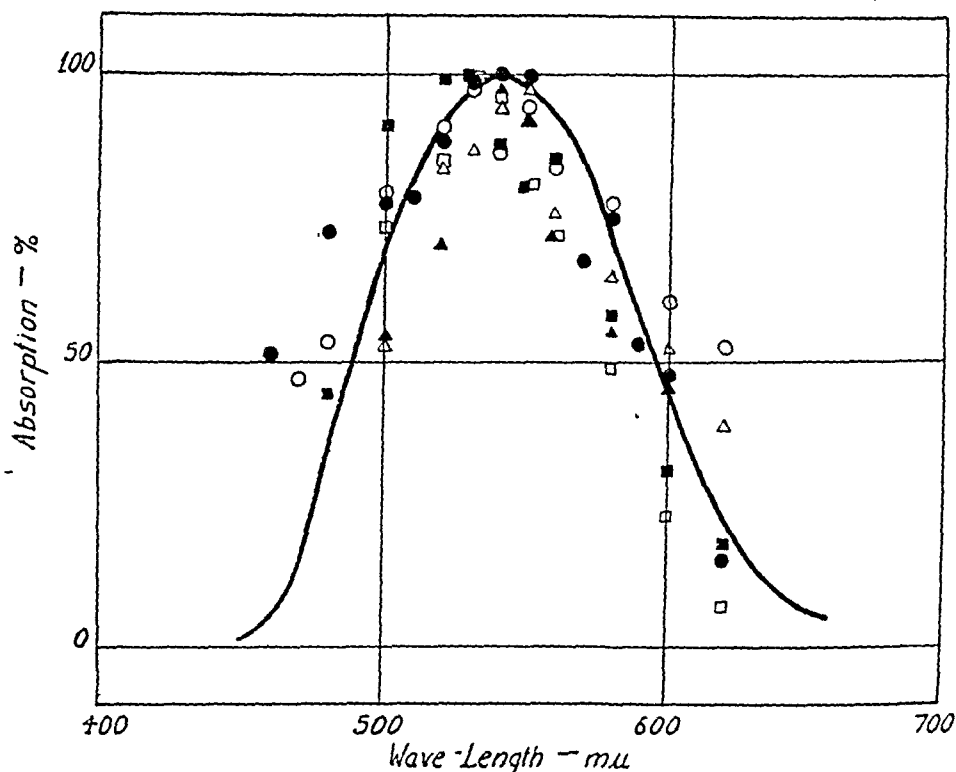


FIG. 6. Absorption spectrum of *Lepomis* visual purple. The points are the individual measurements of Table III; the curve is the average of many measurements of fish visual purple made by Koettgen and Abelsdorff.

This maximum lies between 535 and 545 $m\mu$. The *Lepomis* curve is somewhat narrower than that of other types of fish visual purple, but it is obviously much wider than the visibility curve of the sun-fish.

DISCUSSION

It is to be seen from the data presented in the last two sections that the wave-length of maximum effectiveness for the eye of *Lepomis* is

almost identical with the wave-length which is absorbed most by its visual purple. Taking into consideration the fact that this correspondence also occurs in man, the frog, and the hen, we may conclude that visual purple is the photosensitive substance which determines at least the maximum of the visibility curve measured at low intensities. Thus, one prediction which is made on the hypothesis that visual purple is responsible for dim vision has been verified for an animal whose visual purple is different from that found in all the groups which have been previously studied.

This hypothesis also predicts that the shape of the visibility curve should be determined by the absorption spectrum of fish visual purple. The measurements on *Lepomis*, on the other hand, clearly show that such is not the case. There is, however, good reason for believing that we are dealing with an exceptional case.

The different visibility functions of Animal 8, which have already been mentioned, give a clue to the probable explanation. This animal was measured on three occasions. The first visibility curve obtained on this animal (8I) was very much broader than that usually obtained. Measurements made 3 days later produced a curve intermediate between the first set and the normal. Finally, measurements made 2 weeks later gave a visibility curve which was practically identical with the usual curve. The data obtained in the first and the last measurements on Animal 8 are plotted in Fig. 7. The upper curve is for the last set of determinations and is essentially similar to that shown in Figs. 4 and 5. In the lower half of Fig. 7, the circles represent the data for Set I of Animal 8. The continuous curve is the absorption spectrum of fish visual purple, while the broken line is the right portion of the usual low intensity visibility curve as taken from the curves of a number of other animals. It is evident that the data fit a composite curve very well.

The explanation which is proposed for the distortion of the usual visibility curve of *Lepomis* also takes into account the variable results on Animal 8. The data are explainable on the assumption that the eye of the sun-fish contains besides visual purple probably two light absorbing, but not light sensitive, pigments. Accordingly, the amount of light of any given wave-length which is needed to permit a response by the animal is the sum of the amount which is absorbed by

the visual purple and that which is absorbed by the non-sensitive pigments. This extra absorption by the pigments causes a distortion of

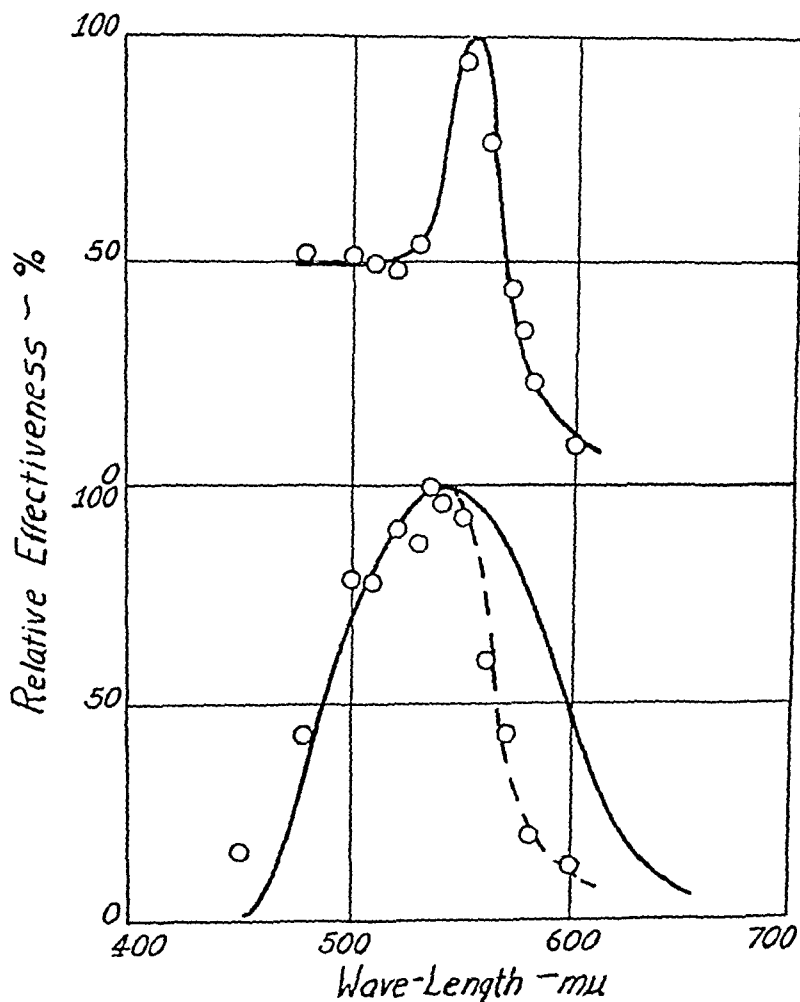


FIG. 7. Visibility function of Animal 8 at two different times. The data in the upper half (8III) were secured 3 weeks after the data in the lower half (8I). The upper data are just like those given by all the other animals. In the lower half the continuous line is the absorption spectrum of fish visual purple according to Koettgen and Abelsdorff while the broken line is the right half of the curve representing the usual low intensity visibility function.

the visibility curve, which is determined primarily by the visual purple alone. The extreme narrowness of the usual visibility curve is probably due to a decrease in the total absorption of the two pigments in

the region of the maximum, which leaves it substantially unaltered and at the same time accentuates it with respect to the remainder of the curve. There is evidence for this from the nature of the pigments which I believe are responsible for the distortion of the visibility curve.

That there are pigments present in the eyes of fish has been known for many years. Cunningham and MacMunn (1893) described the presence of carotinoids. Melanin and guanin had been found even earlier. A discussion of the chemistry and distribution of these pigments is contained in the monograph of Verne (1926). The cornea of the sun-fish eye too, is of a grayish green color, so that it also may act as an absorbing filter.

A series of very careful studies have been recently made on the retinas of bony fish by Wunder (1925). He pictures the guanin and melanin pigments of the retina. Especially noteworthy is his Fig. 16, p. 36, where it is seen that even in the dark adapted eye there is a dispersal of guanin around the rods.

My data indicate at least two pigments, one absorbing to the right and the other to the left of the maximum. They vary in concentration independently, and, since No. 8 showed at one time the complete absence of the pigment absorbing on the left while still possessing the other pigment (which does not seem to vary much from animal to animal), it is probable that this variable pigment is carotin. Carotin has a strong absorption band in the region demanded by the data (McNicholas, 1931) and is a nutritional pigment which is stored and used up as the physiological conditions vary (Palmer, 1922). The day-to-day variations in the left portion of the visibility curve of any one individual is also probably due to this variation in the concentration of carotin. The second pigment, whose absorption is mainly to the right of 550 $m\mu$, may possibly be guanin but no absorption measurements on it have been discovered in the literature.

Such pigments are present in the eyes of most vertebrates; in most humans the quantity is not great enough to change the visibility function materially, though the macular pigment is well known and its effects recognized (Kohlrausch, 1931). Dieter (1929) records one set of measurements which is explainable on the assumption that the subject had a large amount of pigmentation. He obtained the dim vision curve of a normal individual, for comparison with those of hemeralopes, which bears a remarkable similarity to those I have

obtained for fish. Unfortunately, his data are not corrected for the energy distribution of the source, so that no strict comparison can be made.

Another example of the distortion of the visibility function can be seen in Honigsmann's (1921) curves for fowl. Here, the cone curve is like the human, but the rod curve is very much narrower.

The same solution may be applicable to the data of Murr (1928) on the cat. He suggests another possibility, that reflection from the tapetum lucidum is responsible for this deviation.

CONCLUSIONS

The hypothesis that visual purple is the pigment whose reaction to light is the primary cause of vision has been subjected to a test. This hypothesis was put forward on the basis of the correspondence of the absorption spectrum of one type of visual purple with the visibility function of those vertebrates which possess this type of pigment. The test which has been applied takes advantage of the fact that there are two major groups of visual purples, as classified by their absorption spectra. If the hypothesis is correct, the visibility function of an animal possessing the second type of visual purple should be different from that of the animals having the first type, and should be determined by its visual purple.

This hypothesis is considered to have been substantiated adequately by this test, since, in dim vision, the wave-length of maximum effect on the eye of the sun-fish has been found to be *ca.* 540 $m\mu$, and thus is determined by the type of visual purple which is present in its eye.

The visibility function has a different form than can be predicted from a knowledge of the absorption spectrum of fish visual purple. This is considered due to the presence, in *Lepomis*, of pigments which are light absorbent but not light sensitive. These mask the form of the visibility function and, to that extent, may detract from the strength of the proof. It is possible that an investigation of other species of fish, either normal or albino, may reveal the visibility function without this added complication.

SUMMARY

1. A test is proposed of the hypothesis that visual purple is the photosensitive substance concerned in dim vision. It is based on the

fact that fish visual purple is different from that of other vertebrates. If the hypothesis is correct, the fish dim-visibility function should be different from that of other vertebrates and should be determined by the absorption spectrum of its visual purple.

2. A new method is described for obtaining the visibility function of fish, in quantitative terms. It depends on the measurement of the least amounts of various spectral energies which will produce a visual orienting response to the displacement of a constant background.

3. Data are presented on thirteen animals. It is shown that the maximum of the visibility function is identical with the maximum of the absorption spectrum of fish visual purple. The shapes of the visibility curves obtained are, however, variable and different from that of the absorption spectrum.

4. The possibility that *Lepomis* visual purple is different from that of other fish is ruled out by a series of measurements which confirm the results of Koettgen and Abelsdorff on other fish.

5. Reasons are given for the conclusion that there are present in *Lepomis* special conditions which distort the visibility curve out of true agreement with that predictable from the absorption spectrum of its visual purple. The suggestion is made that the presence of light absorbing, but not light sensitive, pigments is responsible for this distortion. One of these pigments may perhaps be carotin while the second is unspecified.

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THE RÔLE OF INTRACELLULAR BACTERIOPHAGE IN LYSIS OF SUSCEPTIBLE STAPHYLOCOCCI

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In a previous paper on the kinetics of the bacterium-bacteriophage reaction (1) it was shown that under certain set conditions lysis of susceptible staphylococci occurs when the total phage *i.e.*, extracellular plus intracellular phage, present in a bacterium-bacteriophage mixture attains a value of 125 activity units (2, 3) per organism, and that lysis is not determined by the total quantity of phage per ml. of suspension.

This is true both for cultures actively growing in the presence of phage and for suspensions in which resting cells are exposed to the critical number of phage units by addition of the requisite amounts of phage.

In a suspension of bacteria containing phage, the phage is distributed in such a way that the concentration of phage in the bacteria is directly proportional to the concentration of phage in the surrounding solution (4). The phage is therefore partly inside and partly outside the bacterial cell and lysis may conceivably depend on any one of the following four conditions.

Total phage per bacterium (P/B), external phage per bacterium (P_e/B), internal phage per bacterium (P_i/B) or external phage per ml. [P_e]. These latter two values are proportional to each other as mentioned above and therefore cannot be separated by any experiment carried out under equilibrium conditions. It is possible however to decide whether or not the total phage per bacterium is the essential condition by diluting a suspension of phage and bacteria containing slightly more than the amount of phage necessary for lysis. When this is done the total phage per bacterium will evidently be constant in all

the dilutions and the external phage per bacterium will increase, whereas the quantity of internal phage per bacterium will diminish as will the quantity of external phage per ml.

If lysis therefore is conditioned by the ratio of total phage, or external phage, to bacteria all the dilutions will undergo lysis, whereas if lysis is determined by the quantity of internal phage per bacterium or by the quantity of external phage per ml. the more dilute suspensions will not undergo lysis. Experiment shows that this latter result is obtained.

The experimental test was carried out as follows: 9×10^{10} Phage Units and 6×10^8 staphylococci (18 hour washed culture) were suspended in a total volume of 10 ml. of broth. 5 ml. of this mixture was added to 5 ml. of plain broth and this in turn was diluted by half. The Phage-Bacterial mixtures, each of 5 ml. volume as in earlier experiments (1, 2, 3) thus represented Total Phage/Total Bacteria ratios of 150 throughout. (It will be noted that the ratio is greater than that found necessary for lysis.) The suspensions were contained in large test tubes of uniform diameter and were placed in a mechanical shaker mounted in a 36°C. water bath. Bacterial growth and lysis were followed by reading turbidities against a standard series made of the same bacteria under conditions of maximal Tyndall effect (1, 2).

The results are shown graphically in Fig. 1 and it is evident that lysis without growth occurred in the original mixture and first dilution only, while cell reproduction with concomitant phage formation took place in the other dilution. The average value of the distribution coefficient, K , was found to be 20×10^3 at 36°C. Using this figure the values of (Pb/B) in each of the three tubes may be calculated from the equation $\frac{Pb/B}{25 \times 10^{10}} = 20 \times 10^3$. They are 124, 111, and 82, respectively.

It would appear then that lysis depends upon the attainment of a threshold > 82 and < 111 phage units within the cell or upon the attainment of the corresponding concentration of external phage per ml.

It is possible to demonstrate the same point in a slightly different manner by adding a small amount of phage to growing bacteria and diluting the culture just before the beginning of lysis. Dilution will not alter the ratio Total Phage/Bacterium but will serve to reduce the value of Pb/B and of $[Pe]$ and it should be possible to decide from the behavior of the suspension after dilution which ratio represents the conditioning factor for cellular dissolution.

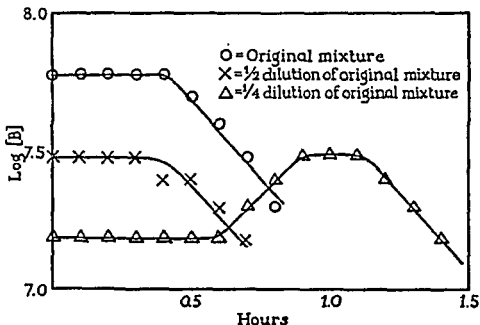


FIG. 1. Lysis depends upon concentration of phage within the cell. Total Phage/Bacterium = 150 throughout. Pb/B decreasing with dilution. Temp. = 36°C .

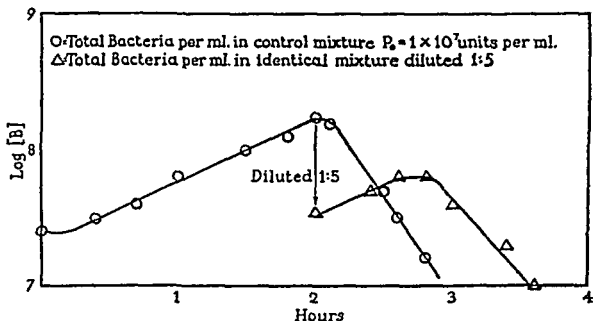


FIG. 2. Effect of dilution at the moment Bacterial Lysis begins on cellular dissolution due to reduction in intracellular Phage concentration. Vol. 5 ml. Temp. = 36°C .

Fig. 2 depicts such an experiment. The initial values of $[P] = 1 \times 10^7$ and $[B] = 2.5 \times 10^7$. A control set was run 0.5 hour ahead of the series to check the calculated t (lysis). Identical sets were run at

intervals of 0.25 hour in order to get several sets of readings after dilution. Just as lysis began each suspension was diluted 1:5 with broth reducing Pb/B from 104 to 83. Turbidity readings showed that lysis in all cases was delayed by dilution until the bacteria had grown, producing more phage, increasing $[Pe]$ and raising the ratio Pb/B to the critical level.

Actual values of Pb/B at the moment of lysis were determined for a number of growing cultures made with widely varying initial $[P]$'s and $[B]$'s as in earlier work (1). The experimental data gave Pb/B ratios ranging from 104–112.

DISCUSSION

The experiments just described show that the significant condition for the occurrence of lysis is either a concentration of about 110 phage units inside each bacterium or of about 12×10^8 units in each ml. of surrounding solution. Since these two quantities are always in constant ratio to each other it is immaterial which one is used. Evidently, also, they cannot be distinguished by any experimental procedure carried out under equilibrium conditions. It is perhaps more reasonable to suppose that the internal phage is responsible for the reaction.

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THE EFFECT OF ADDED LOADS UPON THE GEOTROPIC ORIENTATION OF YOUNG GUINEA PIGS

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I

It has been shown in several instances that mammals, while progressing over a surface inclined at angle α , orient upward in a way such that the relation between the angle of orientation θ and $\log \sin \alpha$ is sigmoid in character, when θ represents the angle at the intersection of an oriented path with the base of the inclined surface. In the cases of young rats (2, 3, 4), and mice (1, 5), the curve is continuous over the entire range of stimulation. A difference appears in the plotted data in the cases of young guinea pigs (7) and adult white rats (6). The functional connection between θ and $\log \sin \alpha$ is again sigmoid in character, but in both instances the curves are discontinuous; each curve is compound, being made up of two sections. In one investigation, including two groups of six and eight young guinea pigs respectively (7), the junctions of the two sections occurred in the region of $\alpha \approx 45^\circ$. In the present investigation the break occurred at a lower inclination, in the region of $\alpha = 35^\circ$. With the adult white rats the disjunction occurred at $\alpha = 55^\circ$ (6). With both young guinea pigs and adult white rats it was observed that within the range of values corresponding to the discontinuous parts of the plotted curves the mode of progression over the plane changed from *walking* to *hopping* (5, 6). Since the distribution of muscular tensions involved in hopping must necessarily differ from that involved in walking, the change in the mode of progression may be regarded as determining the change in the relation of θ to α .

The conditions which determine the change in mode of progression, and therefore the location of the breaks in the curves relating θ to α , are as yet undefined; but it is hoped that by testing animals under the

added load at a given value of α followed the testing of the animal without the load at that inclination. Because fatigue was found to be an influential factor in the performance, at least 30 minutes was allowed to elapse between any two tests of the same animal. Each value of θ in Tables I and II is the average of 40 observations.

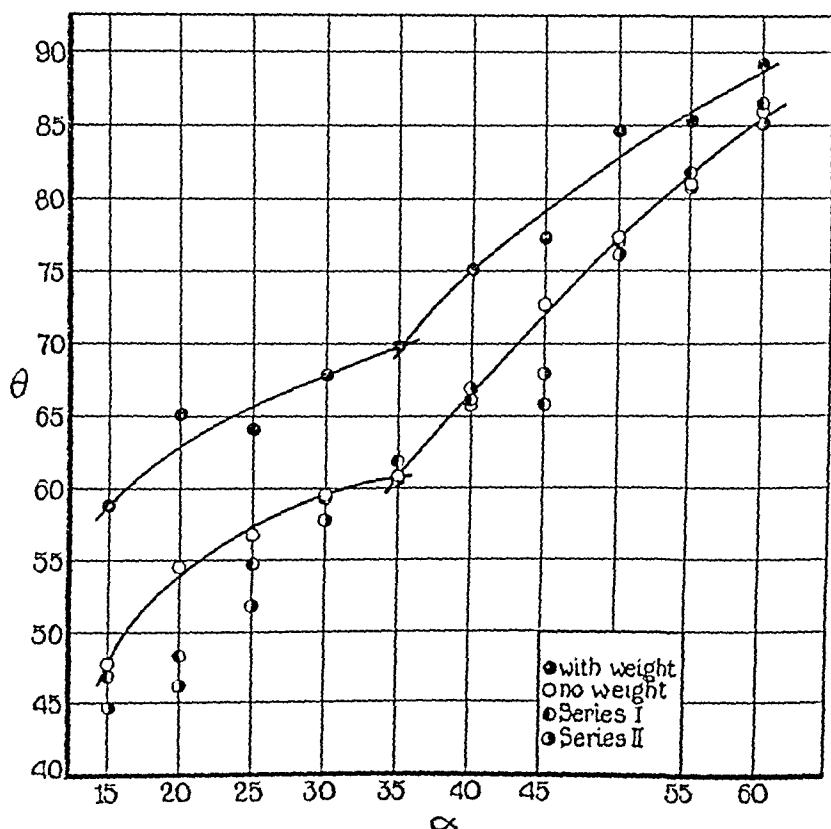


FIG. 1. Angles of orientation *with* and *without* added loads of a group of four animals. The angles of orientation *without* added load, resulting from an earlier experiment (7) involving two groups of animals, have been plotted in this figure to show the agreement between three independent series of observations. In these latter series the breaks in the plotted functions appeared in the region of $\alpha = 45^\circ$.

The mean values of θ are plotted directly against α in Fig. 1. The function which relates θ to α is clearly continuous, and discontinuous factor in

most apparent. At all values of α the θ values are significantly higher for orientation with added load than for orientation without the load. It may be noted that the values of θ become more nearly equal under the two sets of conditions as θ approaches 90° . This is clearly indicated in all the figures and would be a reasonable prediction, since both with and without added load the value of the response approaches 90° as a

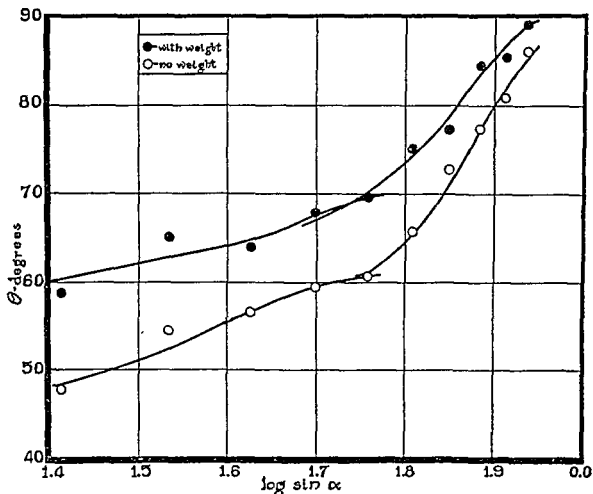


FIG. 2. Angles of orientation (mean θ), for guinea pigs (four litter-mates), *with* and *without* added load. The compound nature of the relation between θ and the independent variable α , and the effect of the added load on orientation, are apparent in this figure.

limiting value. The data collected in an earlier experiment with guinea pigs (7) is also plotted in Fig. 1. The characteristics which are apparent in these earlier series of observations are quite as apparent in the data of the present experiment. The discrepancies between the data of the two earlier series and the data of the present experiment may be due to any of a number of variable factors such as age

and genetic constitution. It is hoped that the influence of such factors upon the geotropic orientation may be more thoroughly investigated in the near future.

In Fig. 2, where θ is plotted against $\log \sin \alpha$, the curve relating the two variables is of the type which constantly recurs in measurements of the sort that we are dealing with here. For both sets of data (*i.e.*, with and without added load) the curves are sigmoid but with a disjunction occurring in the region of $\alpha = 35^\circ$. These observations

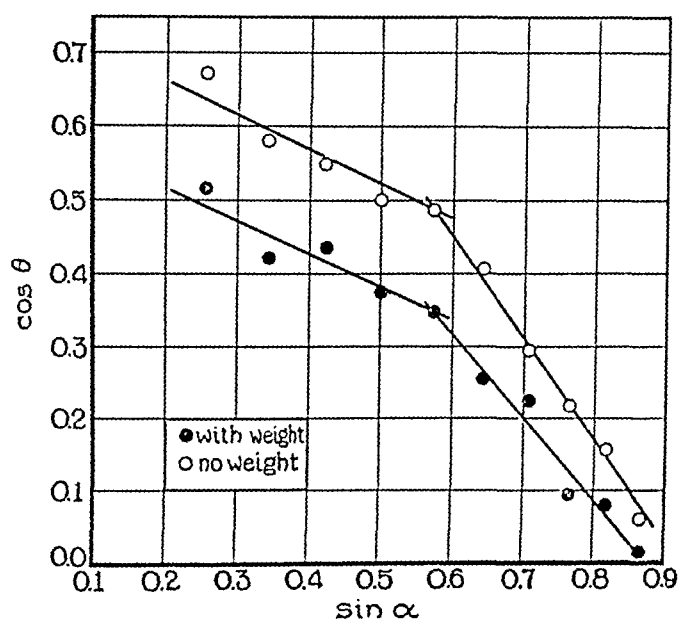


FIG. 3. The cosines of the angles of upward orientation θ , both *with* and *without* added load are directly proportional to $\sin \alpha$. Two regions characterized by different proportionality factors are represented here.

correspond in a general sense with earlier observations on young guinea pigs (7) and adult rats (6), where the curves relating θ to $\log \sin \alpha$ were sigmoid but with the cusps appearing in the regions of $\alpha = 45^\circ$ and $\alpha = 35^\circ$ respectively.

When $\cos \theta$ is plotted against $\sin \alpha$ (Fig. 3) $\cos \theta$ decreases as $\sin \alpha$ increases, and the relation in both sets of data is apparently rectilinear with two regions in which the factor of proportionality differs.

If it is legitimate to regard the variation of θ as being determined by the conditions which determine the extent of orientation (\dagger), a

plot of the variation of mean θ (*i.e.*, P.E. θ with n constant) against $\sin \alpha$ should display the disjunction which is characteristic of the curve relating the magnitude of response to the independent variable. Such a plot (Fig. 4) reveals a relationship which is apparently rectilinear but with a zone of discontinuity in the region of $\alpha \approx 35^\circ$ (3). For equal values of θ , P.E. θ is practically identical under the two sets of conditions.

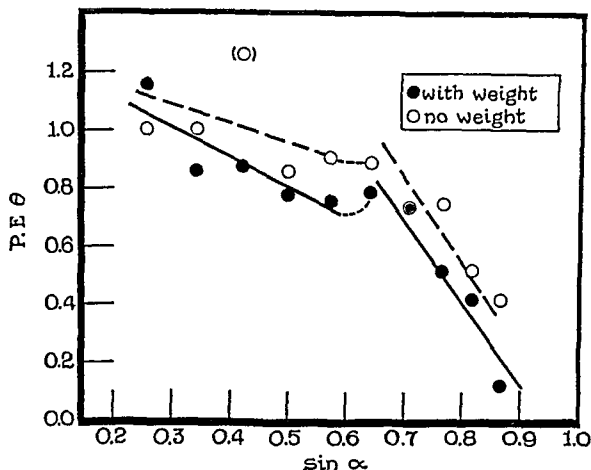


FIG. 4. Probable errors of mean θ as a function of the sin of the angle of inclination α . There were 40 observations at each slope of the inclined surface. The apparently aberrant point enclosed in parentheses was not given equal weight with the rest of the points in fitting the line.

SUMMARY

It has been found that young guinea pigs when progressing over a surface inclined at an angle α with the horizontal, orient upward in a way such that the path described by an oriented animal is at a mean angle θ with the base of the inclined surface. The magnitude of mean

lating θ and α is compound, being made up of two sections with a break which corresponds to a change in the mode of progression of an animal over the surface of the plane.

If a load of constant relative mass¹ is attached to the back of an animal, midway between the fore and hind legs, testing on an inclined surface reveals the fact that the magnitude of mean θ is increased over the entire range of stimulation, with the two values of mean θ (*i.e.* with and without added load) becoming more nearly equal as they approach 90° as a limiting value. The variation (P.E. of mean θ) is not sensibly changed by attaching a load to an orienting animal; for equal magnitudes of θ , under the two sets of test conditions, the P.E.'s are very nearly equal.

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¹During the time which elapsed while the experiment was in progress the body weights of the four animals gradually increased so that it was necessary to increase the weights of the added loads each day to keep them equal to 10 per cent of the body weights of the animals. The loads were within the ranges which follow: Animal 1, 12.5 to 17.0 gm.; Animal 2, 11.8 to 17.0 gm.; Animal 3, 13.2 to 18.4 gm.; Animal 4, 12.0 to 17.4 gm.

THE EFFECT OF DENATURATION ON THE VISCOSITY OF PROTEIN SYSTEMS

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Concentrated solutions of proteins in acid or alkali may become very viscous on denaturation of the protein and under suitable conditions a moderately viscous solution can be converted by heating into a clear gel. These striking changes in viscosity, although they have been known for a long time, have not been studied in detail in recent years and in general the study of denaturation has been neglected. The main reason for this neglect has been the belief that denaturation is an irreversible reaction, probably an irreversible splitting up of the protein, and that therefore denaturation is of little physiological interest. The experiments described in previous papers (reviewed in Anson and Mirsky, 1931a) led us to the conclusion that the denaturation and coagulation of proteins, contrary to the usual notion, are reversible; and that therefore denaturation is a biologically possible process. We accordingly became interested in resuming the older investigations of the gross change in viscosity accompanying denaturation. The present experiments make more precise than was possible a generation ago the conditions for obtaining very viscous solutions and gels, and they show that one can convert not only a concentrated protein solution but even a solution containing only one per cent of protein into a clear gel. Although, as will be seen, there is an increase in viscosity due to denaturation itself apart from any aggregation of the molecules, in the very viscous solutions containing little protein the denatured protein seems to be in the form of aggregates, invisible precipitates, which occlude water.

Some of the biological processes, such as muscular contraction, which there is some reason to believe are accompanied by denaturation are also accompanied by a gross increase in viscosity. It might be supposed that the viscosity changes are a result of denaturation. If, however, the viscosity change due to denaturation is, in general, due to aggregation, then since aggregation is not peculiar to denatured proteins, an increase in viscosity cannot by itself be taken as proof of denaturation. The proof that denaturation occurs biologically must come from tests more specific for denaturation.

The experiments on the viscosity of denatured egg albumin solutions provide a possible explanation of the difficulties encountered in the reversal of the denaturation of egg albumin. The essential step in the preparation of native protein from an acid solution of denatured protein is neutralization. But neutralization also causes precipitation of a denatured protein and if the protein is precipitated reversal of denaturation is prevented. With the relatively soluble denatured serum albumin one can get reversal without any particular precaution to avoid precipitation (Anson and Mirsky, 1931c). With the less soluble denatured hemoglobin and globin, rapid complete neutralization causes complete precipitation and no reversal takes place. To obtain reversal the neutralization is stopped before enough alkali has been added to cause visible precipitation (Mirsky and Anson, 1929, 1930; Anson and Mirsky, 1930, 1931b). With the very insoluble denatured egg albumin it is still more difficult to reach the reversal conditions without the protein becoming aggregated. The viscosity experiments show that denatured egg albumin becomes aggregated under the very conditions under which denatured hemoglobin is converted into native hemoglobin. In harmony with this result, only recently and under favorable conditions have we been able to reverse the denaturation of egg albumin at all and then only to a small extent (unpublished experiments).

EXPERIMENTAL

The egg albumin used in these experiments was thrice crystallized and finally dialyzed in a shaking dialyzer (Kunitz and Simms, 1928) against distilled water at 5°C. A 10.3 per cent solution had a conductivity of 5.1×10^{-3} reciprocal ohms, a value which shows the needlessness of electrodialysis.

To prepare the ox hemoglobin used, laked red blood corpuscles were diluted

with an equal volume of water, shaken with a fifth the total volume of toluol and allowed to stand in the cold. The next morning the toluol and swollen stromata were removed by centrifugation.¹

Relative viscosity of centrifuged solutions was measured with an Ostwald viscosimeter. A few measurements made with the du Noüy coaxial cylinder viscosimeter gave essentially the same results.

Effect of Acid.—In the first series of experiments (see Table I) solutions of 4 per cent egg albumin containing various concentrations of HCl are heated for 4 minutes in large test tubes kept in boiling water and then cooled to 25°C. If not enough acid is added the protein is precipitated. If just enough acid is added to prevent precipitation the

TABLE I
Effect of pH on Viscosity of Heated 4 Per Cent Albumin

HCl <i>mols per liter</i>	pH	Appearance	Relative viscosity
0.004	4.29	Precipitate-gel	
0.005	3.96	Slightly opalescent— almost a gel	
0.008		Clear	8.08
0.010	3.70	Clear	3.92
0.012		Clear	2.09
0.040		Clear	1.52
0.065		Slightly opalescent	About 40

solution is very viscous. The more acid the solutions the less viscous they are after being heated and then cooled, until finally further addition of acid makes them more viscous again and slightly opalescent. As in other protein phenomena, after a certain point the addition of acid has the same effect as the addition of salt. It may be seen in these as in other experiments, that small differences in hydrogen ion concentration may be accompanied by great differences in viscosity, especially

¹ All the experiments described in the present paper, except the one on the hydration of hemoglobin in urea solution, were done in the winter of 1927-28. Since then we have found it desirable to use alumina cream to facilitate the removal of toluol and stromata. The remark is made to avoid giving the impression that the use of alumina cream described in other papers has been abandoned. It may be that the commercially available Filter-cel which is very useful in the filtration of some protein solutions can be substituted for alumina cream.

in the range near the precipitation zone where the solutions are very viscous.

Effect of Salt Added before Heating.—In the previous section there was described a heated solution of 4 per cent albumin in 0.012 *N* HCl which had a relative viscosity of 2.09. If NaCl is added before the heating so that its concentration is 0.005 *M* then after the heating the solution is more than three times as viscous as without the salt (see Table II). The same effect is produced by 0.0002 *M* K₂SO₄ and by even smaller concentrations of K₄Fe(CN)₆. Similar experiments can be done in great variety with the general result that whenever a moderate increase in viscosity can be caused by heating in the absence of salts and the presence of a small concentration of salt results in visible

TABLE II
Effect of Salt on Viscosity of Heated 4 Per Cent Albumin

Salt	Concentration	Time after heating	Relative viscosity
		<i>hrs.</i>	
NaCl	0.005 <i>M</i>	0	7.3
		2	11.8
		4	13.4
		22	17.4
K ₂ SO ₄	0.002 <i>M</i>	0	7.3
		2	10.3
		4	11.5

precipitation of the protein, then a still smaller concentration of salt can be found which can cause a great increase in viscosity on heating.

It is characteristic in these experiments that the viscosity of a heated self-containing solution gradually increases with time. Thus the solution containing 0.005 *M* NaCl in 22 hours reaches a viscosity almost nine times as great as that obtained if no salt is added.

Effect of Salt Added after Heating.—If salt is added to a suitable solution of albumin which has been heated and then cooled the viscosity rises and continues to rise for a long time. The gradual rise in viscosity can be speeded up and made more extreme by raising the temperature. For instance, a solution whose relative viscosity is only 1.55 is obtained by adding an equal volume of water to a 3 per cent solution of

albumin in 0.006 N HCl which has been heated 4 minutes and cooled to 25°C. When an equal volume of 0.002 M K_2SO_4 is added to the solution the viscosity is 2. After 22 minutes it is 2.76 and after 2 days the solution has become a slightly opalescent hard gel. A hard gel may be obtained in 30 seconds by placing a test tube of solution containing 0.001 M Na_2SO_4 in boiling water.

A similar experiment illustrates the facts (1) that one can obtain viscous solutions whose protein content is only 1 per cent and whose salt concentration is only 0.0008 M, and (2) that whether or not protein is precipitated when its solution is heated depends not only on the composition of the solution but also on its history. An equal volume of 0.0016 M K_2SO_4 is added to a heated 2 per cent solution of protein in 0.004 N HCl. The viscosity is 1.28 and increases gradually. Warming to 37°C. causes a gross increase in viscosity and boiling in boiling water for 3 minutes makes the solution slightly opalescent and almost a gel. If an equal volume of 0.0016 M K_2SO_4 is added to an unheated 2 per cent solution of albumin in 0.004 N HCl, on being heated the protein is definitely precipitated.

Effect of Alkali Added after Heating in Acid.—If a solution of albumin in acid is heated and then neutralized the protein is precipitated. However, the amount of alkali added is just not enough to cause precipitation then the resulting solution in time becomes very viscous if the concentration of protein is low. For example, to a heated 2 per cent solution of albumin in 0.006 N HCl is added an equal volume of 0.00268 N NaOH. The viscosity at first is 1.67. After 19 hours it is 6.82.

Effect of Addition of Native Protein.—The addition of native, salt-egg albumin to albumin heated in acid has the same sort of effect as the addition of NaOH. The solutions become more viscous and the viscosity continues to rise with time. Very characteristic in these solutions is the great increase in viscosity produced by warming the solutions only a few degrees. The native albumin probably acts not only by combining with the acid to produce a less acid solution in which the denatured albumin is less soluble, but also by combining with the denatured albumin itself, since, at the pH produced, denatured albumin would precipitate in the absence of native protein. In general, in many so-called mixtures of soluble and insoluble proteins of

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all sorts when the conditions are such that the insoluble protein does not precipitate, although it would precipitate in the absence of the soluble protein, then the solution of the two proteins may have a variety of physical properties not possessed by solutions of either of the two proteins taken alone.

The experiment consists in adding to a 3 per cent solution of albumin heated in 0.006 N HCl a 10.3 per cent solution of dialyzed native albumin. When an equal volume of native albumin is added, the viscosity is at first 1.73 and the next morning 7.50. When a third more native albumin is added the viscosity is at first 1.75 and the next morning too great to be measured.

Effect of Water Added before and after Heating.—If an acid solution of albumin is diluted with water before it is heated, the water has a much greater effect in lowering the viscosity than if it is added after the heating of the more concentrated solution. For instance, if a 4 per cent solution of albumin in 0.008 HCl is diluted with an equal volume of water before heating, then after heating the viscosity at 25°C. is 1.48. If the dilution is done after the heating the viscosity is 3.85. Even more striking results can be obtained by using somewhat more viscous solutions. It is easy to obtain two solutions of the same composition whose viscosities differ more than five times.

Acid likewise has a greater effect on the viscosity when it is added before rather than after the heating. As has already been shown, near the precipitation zone the addition of a little more acid before the heating makes the viscosity on heating much lower. The addition of the same amount of acid after heating has little more effect than the addition of the same amount of water.

Without multiplying examples it may be said that in general it is easier to prevent the formation of a viscous solution by adding water, acid or alkali, than to decrease grossly the viscosity of an already viscous solution, just as it is often easier to prevent the precipitation of a substance than to dissolve it once it is precipitated. Heat coagulated and precipitated albumin does not dissolve readily in a concentration of acid which is sufficient to prevent the precipitation on heating.

Effect of Urea.—The addition to saturation of solid urea to a very viscous solution of denatured egg albumin always results in a gross lowering of viscosity. For instance, 4 gm. of neutral urea were added

to 5 cc. of a solution whose viscosity thereupon dropped from 8.18 to 2.14. Furthermore in many cases the viscosity is about the same whether the urea is added before or after the heating. It is impossible to get a very viscous solution of denatured albumin in saturated urea solution with the concentrations of proteins and electrolytes used in the experiments already described.

The experiments with egg albumin can be varied in many ways, always with the result that if only one factor is varied at a time then a range can be found in which the viscosity is very sensitive to that factor. It is in this range, which is always close to the precipitation zone, that the highest viscosities are obtained.

Experiments similar to those with egg albumin and giving similar results can be done with other denatured proteins. A few experi-

TABLE III
Effect of pH on Viscosity of Heated 5 Per Cent Hemoglobin

HCl	Relative viscosity
<i>mols per liter</i>	
0.0021	7.60
0.0024	3.18
0.0063	2.37

ments with hemoglobin are summarized in Table III. The hemoglobin concentration is 5 per cent, the time of heating 4 minutes. The solutions are filtered before the viscosity measurements and are all clear.

DISCUSSION

Viscosity Change Due to Aggregation.—All the theories of viscosity agree that the viscosity of a solution depends in some way on the fraction of the total volume occupied by the solute. Kunitz (1926) has shown empirically that when partial volume is plotted against viscosity the same curve results for a number of different colloids. By the use of this curve it is possible to calculate in a new case the partial volume of the solute from the viscosity of the solution. When the value so obtained is higher than that calculated from the concentration and

density of the solute, then one must assume that the solute contains water. When the amount of water is greater than can reasonably be attributed to ordinary hydration then one must further assume that the solute is not dispersed into molecules of the ordinary size but that it has a structure which consists of aggregates occluding water. Such *a priori* considerations lead to the view that in the more viscous solutions of denatured protein the protein is in part aggregated. This view is in harmony with all the experimental facts.

1. In general the conditions for a high viscosity are close to those for visible precipitation. If an acid solution of heated, denatured protein is brought just a little closer to the isoelectric point or a little salt is added, the solution becomes much more viscous or the protein actually precipitates. If the solution before being heated is slightly further away from the isoelectric point or contains slightly less salt then the viscosity of the heated solution is very much reduced. It is characteristic of colloidal aggregates that in a narrow critical range slight changes in ionic concentrations should cause either further aggregation or dispersion.

2. The great increase in viscosity which follows the heating of a suitable protein solution can readily be prevented by the addition of water or acid *before* the heating. The addition of water or acid *after* the heating has much less effect on the viscosity. It is known that it is easier to influence the formation of aggregates and precipitates than it is to dissolve them once they are formed.

3. Urea which can dissolve denatured proteins always lowers the viscosity of a very viscous solution of denatured protein. Were there no solubility effect, the addition of solid urea ought to increase the viscosity.

4. The increase of viscosity with time in many of the solutions can be accounted for by gradual aggregation.

Viscosity Change Not Due to Aggregation.—Although the gross increase in viscosity, which may under suitable conditions accompany the denaturation of a dilute solution of protein, is probably due to aggregation of the protein, there is evidence that denaturation of itself causes an increase of viscosity apart from any aggregation. Concentrated solutions of urea not only dissolve coagulated proteins (Spiro, 1900; Ramsden, 1902) but also denature native protein (Ramsden,

1902, 1913; Anson and Mirsky, 1929; Hopkins, 1931). Denatured protein precipitates when the urea is removed. The more the protein is denatured as determined by the solubility test the more viscous the solution becomes. When the protein has become completely insoluble at its isoelectric point in the absence of urea, the viscosity ceases to change (Anson and Mirsky, 1929). The final relative viscosity of an 8.5 per cent solution of hemoglobin may be 6.3. Since these experiments were described, Burk and Greenberg (1930) and Huang and Wu (1930) have shown by osmotic pressure measurements that denatured proteins are *not* aggregated in concentrated urea solution. The molecular weight of denatured hemoglobin in urea solution is not greater than the molecular weight of native hemoglobin in water. Burk and Greenberg, however, in order to make the osmotic pressures of solutions of different protein concentrations proportional to the protein concentrations had to assume that hemoglobin is hydrated to the extent of 2.8 gm. of water per gm. of protein. Kunitz (1927) had previously calculated the hydrations of gelatin at different concentrations needed to make the osmotic pressure of gelatin solutions proportional to the protein concentration, and he had shown that the same values for the hydration could be calculated independently from the viscosities of gelatin solutions by the use of his curve which relates viscosity to solute volume. We have now likewise shown that the hydration of denatured hemoglobin in urea solution calculated from the viscosity of the solution is about the same as that calculated by Burk and Greenberg from their osmotic pressure measurements which showed denatured hemoglobin in urea solution not to be aggregated. The increase in the viscosity of a concentrated urea solution of hemoglobin as the protein denatures is thus probably due to ordinary hydration and not to aggregation. This increase in viscosity must not be confused with the *decrease* in viscosity which is obtained when solid urea is added to the very viscous solutions of egg albumin. In such cases the urea is dissolving aggregates of already denatured protein.

A solution containing 0.385 gm. denatured dialyzed horse hemoglobin, 5.5 gm. urea and 10 ml. water has at 30°C. a viscosity of 1.6 relative to the viscosity of a solution containing 5.5 gm. urea per 10 ml. water. From Kunitz's curve one can read off that the solvated hemoglobin occupies a volume of 10 per cent of the total volume or 1.44 ml.

Since 0.385 gm. non-solvated hemoglobin occupies 0.29 ml., the solvation per gm. of hemoglobin is $\frac{1.44 - 0.29}{0.385}$ or 3.0 ml. This calculation involves the assumption that so far as using Kunitz's curve is concerned, a solution of hemoglobin in urea solution can be treated the same as a solution of hemoglobin in water. One can obtain about the same value for the solvation of the hemoglobin without using Kunitz's curve by finding what volume of sugar has to be substituted for the solid hemoglobin in the urea solution to get the same viscosity.

CONCLUSIONS

The viscosity of a protein solution is increased by the denaturation of the protein. This is true both when there is the formation of protein aggregates which occlude water and when there is no aggregation. Under certain conditions, as a result of the aggregation following denaturation, a solution containing only one per cent of protein may be converted into a clear gel. The conditions for obtaining very viscous solutions containing little denatured protein are always close to the conditions for actual precipitation and under these conditions the viscosity is very sensitive to slight changes in the concentration of salts and hydrogen ions.

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THE KILLING OF COLON BACILLI BY ULTRAVIOLET LIGHT

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The experiments described in this paper are measurements of the survival ratios of *B. coli* irradiated for different lengths of time by monochromatic ultraviolet light. As such they are an extension to the ultraviolet region of observations previously made with cathode rays¹ and with X-rays.²

There are several qualitative investigations of the destructive action of ultraviolet radiation upon bacterial cells. A quantitative study³ also has been published of the killing of *S. aureus* by a number of ultraviolet wave lengths. When plotted on a proper scale the resultant death curves are of a multiple-hit-to-kill type. These curves give incorrect data unless each cell is sufficiently separated from its neighbors so that it can produce an independent colony. The well known tendency of staphylococci to cling together in chains makes such a spread almost impossible to achieve. Suitable irradiations of the motile *B. coli* accordingly may be expected to yield a better index of the course of bacterial death.

Technique

The culture was obtained from the American Type Culture Collection. Its cultivation and the method of spreading dilute suspensions of the organisms on agar surfaces are those used in the earlier experiments.⁴ Because the areas that could be irradiated were smaller, however, the concentration of the seeded cells has been somewhat increased.

¹ Wyckoff, R. W. G., and Rivers, T. M., *J. Exp. Med.*, 1930, 51, 921.

² Wyckoff, R. W. G., *J. Exp. Med.*, 1930, 52, 435, 769.

³ Gates, F. L., *J. Gen. Physiol.*, 1929-30, 13, 231, 249; 1930-31, 14, 31.

⁴ Wyckoff, R. W. G., and Rivers, T. M., *J. Exp. Med.*, 1930, 51, 921.

Intense ultraviolet light of a single wave length was obtained from a large quartz mercury vapor lamp and a special monochromator. For measurements of survival ratios it is obviously of the greatest importance that the radiation be of constant intensity throughout an experiment. The current for the lamp therefore was drawn from a large 220 V storage battery. In addition the arc was operated for at least an hour before use to insure that it was running steadily. The total output of light was controlled during every experiment by adjusting, when necessary, the current through the lamp so as to give constant readings from a conveniently placed quartz sodium photoelectric cell.

Statistically significant data will be obtained only when the irradiations are short compared with the organism's reproductive cycle. To obtain such an intense monochromatic ultraviolet beam of sufficient cross section a monochromator of great light-gathering power is required. The one used in the present experiments had quartz lenses 6 inches in diameter and prisms of corresponding size. With it an area, 3×30 mm., could be flooded with a single spectral line by the proper adjustment of its position and that of the slit. As in earlier experiments, this area was defined and marked on the irradiated surface by cutting edges attached to the spectrometer and set in a screen capable of shielding the rest of the agar plate. Several exposures were made on a single seeded plate. Counts of survivors on these areas compared with the number of colonies growing out on a similarly stamped but not irradiated control area gave survival ratios. Failure to have a beam of equal intensity over the entire exposed field represents one of the greatest sources of error in such experiments. In the present instance this was checked by a fluorescent screen and more sensitively by the requirement that different parts of the same field should give like amounts of destruction.

For each experiment the energy flux in the beam striking the irradiated organisms was found by a thermopile which replaced the cutting edge and lay in the plane of the agar surface. This thermocouple was calibrated with a carbon lamp of known energy output obtained from the U. S. Bureau of Standards.

Experimental Results

Survival ratios measured for each of the wave lengths 3132 Å, 2900 Å, 2803 Å, 2699 Å, 2652 Å and 2536 Å are recorded in Table I and plotted in Figs. 1-5. Every ratio is the average of counts on at least 10,000 organisms. It is evident that, with the possible exception of the data from 3132 Å, all these results can be closely represented by straight lines plotted on semilogarithmic paper.

In Table II the effective radiant energies are stated as the amounts needed to kill half the bacteria upon which they fall. They are to be taken as approximate only. Precise estimates require the exercise of considerable care—more than is warranted by the present accuracy of

the biological results. The previously recorded⁵ lethal doses for *B. coli* are of the same order of magnitude but for most wave lengths they are less than those of Table II. Bacteria of different strains were used and the irradiations were carried out under such unlike experimental conditions that it is not evident wherein lies the cause of this discrepancy.

TABLE I
Survival Ratios of Bacteria Irradiated with Ultraviolet Light

<i>B. coli</i>					
Time	Wave length				
	3132 Å	2900 Å	2803 Å	2699 Å	2536 Å
<i>sec.</i>					
5	0.977	0.926	0.900	0.882	0.884
10	0.945	0.808	0.795	0.756	0.793
20	0.907	0.632	0.621	0.536	0.596
40	0.794	0.396	0.422	0.251	0.305
60	0.697	0.236	0.266	0.132	0.191
80	0.601	0.162	0.187	0.082	0.111
100	0.459	0.124	—	—	—
Energy incident per mm. ² per sec.	47.8 ergs	11.3 ergs	7.5 ergs	7.8 ergs	8.0 ergs
<i>B. aertrycke</i>					
10	0.949	—	0.818	—	—
40	0.799		0.326		
80	0.644		0.102		
Energy incident per mm. ² per sec.	46.3 ergs		8.2 ergs		

A few experiments have been made with *B. aertrycke* to compare their killing with that of *B. coli*. The data of Table II and of Figs. 1-5 indicate that these two organisms cannot thus be distinguished. Their equal sensitivity, already found with X-rays, is more striking with ultraviolet light because it must mean practical identity in the specific absorption of their protoplasts for the wave lengths used.

⁵ Gates, F. L., *J. Gen. Physiol.*, 1929-30, 13, 231, 249; 1930-31, 14, 31.

Analysis

The foregoing experiments indicate that colon bacilli under ultraviolet irradiation, just as through the action of X-rays and electrons, die at a rate which is semilogarithmically linear. For cathode rays such a result was interpreted in terms of probability theory⁶ to show that practically every high speed electron absorbed by a bacterium was lethal. The same kind of interpretation applied to the data from soft X-rays led to the plausible explanation that though cell death

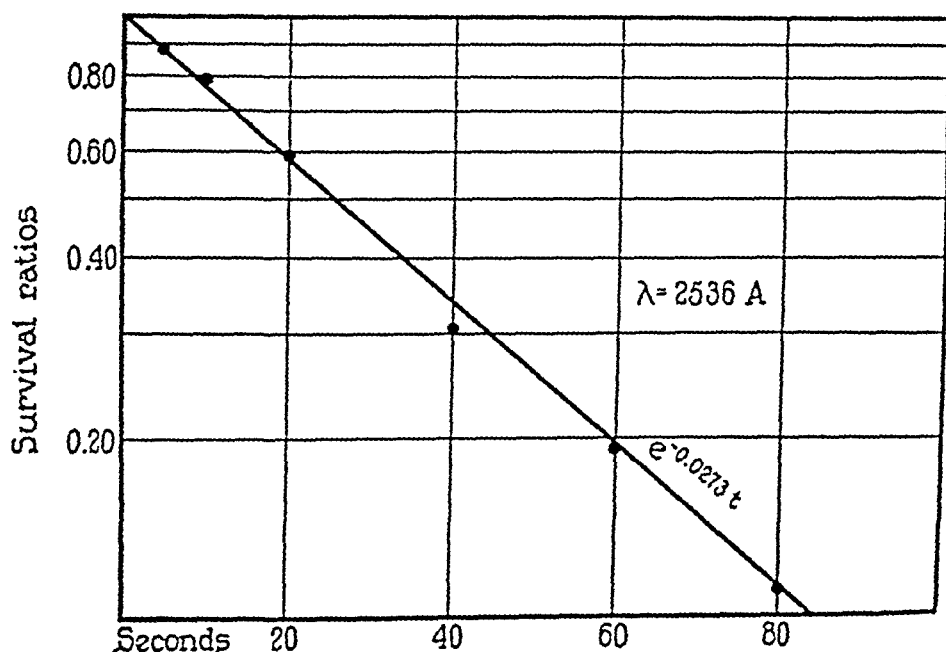


FIG. 1. Survival ratios of *B. coli* irradiated with ultraviolet light of wave length 2536 Å.

takes place through the absorption of a single quantum of energy, this absorption to be deadly must occur within an especially sensitive volume which cannot be greater than 1 per cent of that of a colon bacillus.

⁶ Crowther, J. A., *Proc. Roy. Soc. London, Series B*, 1926, 100, 390; Condon, E. U., and Terrill, H. M., *J. Cancer Research*, 1927, 11, 324; Holweck, F., *Compt. rend. Acad.*, 1929, 188, 197; Rahn, O., *J. Gen. Physiol.*, 1929-30, 13, 179, 395; Wyckoff, R. W. G., and Rivers, T. M., *J. Exp. Med.*, 1930, 51, 921.

It is instructive to carry through a similar analysis based on the results of ultraviolet killing. This will be done using the data from $\lambda = 2699 \text{ \AA}$. The survival ratios A/A_0 of Table I and Fig. 2 follow the equation $A/A_0 = e^{-0.0325t}$. In the language of a quantum interpretation⁷ the average number of effective absorptions per bacterium per second therefore is 0.0325. The thermocouple measurements have shown that in this experiment the energy incident per second is

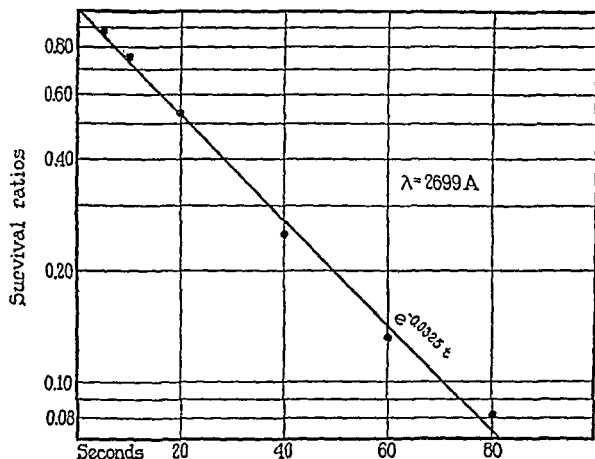


FIG. 2. Survival ratios of *B. coli* irradiated with ultraviolet light of wave length 2699 \AA .

7.79 ergs per sq. mm. Taking the colon bacillus to be a rod 0.5μ in diameter and 2μ long the energy striking a single bacterium will then be 7.79×10^{-6} ergs/sec. The absorption of a thin film of *B. coli* has been measured⁸ for several wave lengths. As in the X-ray experiments it is sufficiently accurate to consider the absorption of a

⁷ Wyckoff, R. W. G., and Rivers, T. M., *J. Exp. Med.*, 1930, 51, 921.

⁸ Gates, F. L., *J. Gen. Physiol.*, 1929-30, 13, 231, 249; 1930-31, 14, 31.

single organism as equivalent to that of a block of protoplasm $0.5 \times 2\mu$ in cross section and 0.42μ thick. Since the absorption of wave length 2699 A in such a layer is 0.127, it follows that the radiant energy absorbed per bacterium per second is 0.99×10^{-8} ergs. The voltage equivalent of the 2699 A line is 4.557 volts. Converting to appropriate units, the $h\nu$ value found from the usual quantum relation $Ee = h\nu$ is directly calculated to be $7.25_s \times 10^{-12}$ ergs. Hence the average number of quantum absorptions per bacterium per

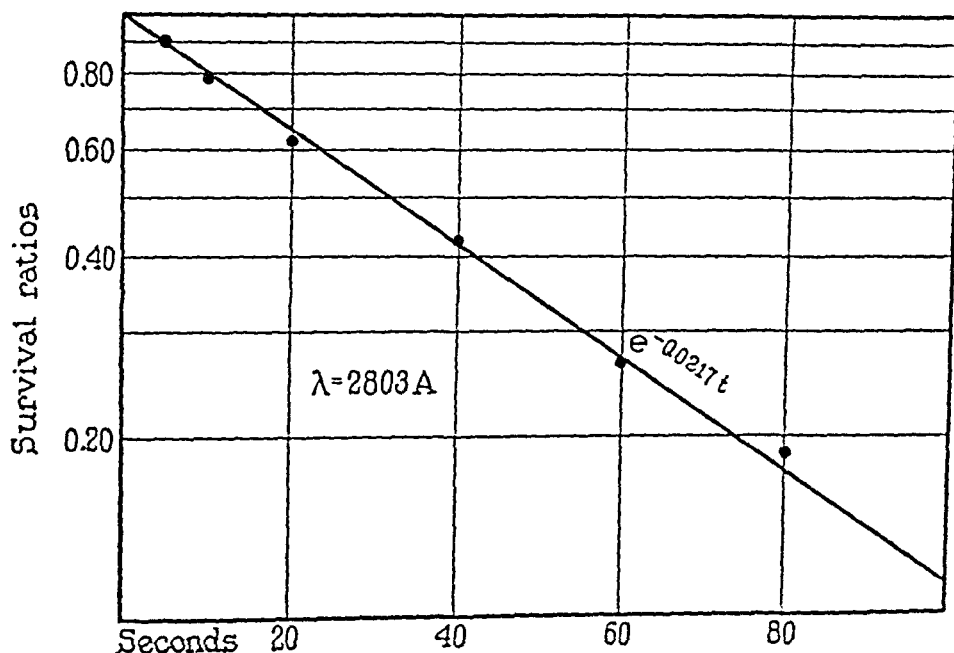


FIG. 3. Survival ratios of *B. coli* irradiated with ultraviolet light of wave length 2803 A.

second is $\frac{0.99 \times 10^{-8}}{7.25 \times 10^{-12}} = 136,300$. Because the average number of absorptions that are effective is 0.0325 per sec., only $\frac{0.0325}{136,300}$, or one in 4.19×10^5 , of the absorbed quanta is capable of causing cell death.

This interpretation may be completed by comparing the "sensitive volume" provided by these calculations with the size of possible structures within an irradiated bacillus. In doing this it must always be borne in mind that what is really being measured is a purely physical

quantity—the sphere of influence within which the quantum acts. The result may be biologically significant in that it sets a definite upper limit to the size of those elements whose injury or destruction results in death. For the present experiments the “sensitive volume” is $\frac{0.0325}{136,300} \times 3.93 \times 10^{-13}$ cc. (the volume of one bacterium), or 9.38×10^{-20} cc.

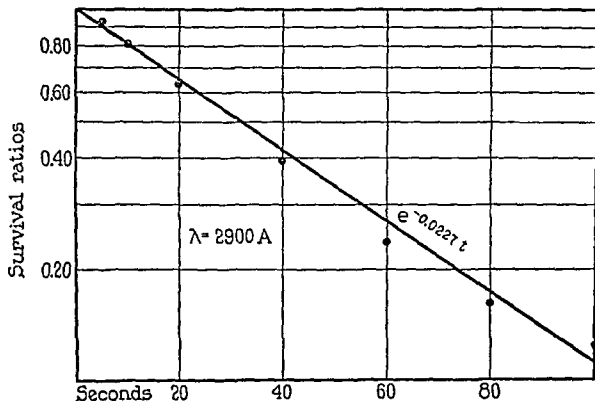


FIG. 4. Survival ratios of *B. coli* irradiated with ultraviolet light of wave length 2900 Å.

A single protein molecule has approximately this size. Neglecting hydrogen atoms to permit calculation, the average atomic weight of a typical protein is not far from 15. One with a molecular weight of 120,000 will thus contain about 8,000 atoms. Assuming the molecule to be cubic it would have 20 atoms on a side. The interatomic distances in organic crystals vary between *ca* 1 Å and *ca* 3 Å. Taking the intermediate value of 2 Å, the protein molecule 40 Å on a cubic side would have the volume 6.4×10^{-20} cc. For longer wave lengths than 2699 Å the calculated “sensitive volume” becomes even less than such molecular volumes. For 3132 Å it is only 1.5×10^{-20} cc.

It should therefore be concluded that if the lethal action of ultraviolet light upon *B. coli* is to be explained in terms of a quantum-hits-to-kill mechanism, the sensitive unit whose well-being is essential to the cell's continued growth and multiplication cannot be larger and may be much smaller than an aggregate of two or three protein molecules of moderate size.

Further data bearing upon the destructive action of ultraviolet radiation can be obtained through comparisons of the amounts of absorbed energy required to kill with ultraviolet light of different wave lengths and with X-rays. Table II records the ultraviolet energies absorbed when 50 per cent of the irradiated organisms are destroyed. These quantities would be expected to be equal only in case killing were directly proportional to the amount of absorbed

TABLE II

Approximate Energies Necessary to Kill 50 Per Cent of Irradiated Colon Bacilli

Wave length	Incident energy	Energy absorbed/bacterium
2536 Å	200 ergs/mm. ²	2.75×10^{-5} ergs
2652	110	1.50 "
2699	160	2.10 "
2803	240	2.50 "
2900	340	2.30 "
3132	5200	10.9 "

energy or in the highly improbable event that the "vital material" of the cell had exactly the same ultraviolet absorption curve as the entire organism. Nevertheless they are of the same order of magnitude. In one of the previous experiments⁹ with copper K series X-rays, 0.22₃ quanta were absorbed per bacterium per second. Since the $h\nu$ equivalent of these X-rays ($\lambda = 1.537$ Å) is $1.27_3 \times 10^{-3}$ ergs, the average energy absorbed per cell per second is directly $0.22_3 \times 1.27_3 \times 10^{-3} = 2.91 \times 10^{-3}$ ergs. Under these circumstances 50 per cent killing is achieved in 48 seconds. The energy to produce this killing, $2.91 \times 10^{-3} \times 48$ ergs = 1.40×10^{-7} ergs absorbed per bacterium, is little more than one per cent of that required to produce the same effect in the ultraviolet region.

⁹ Wyckoff, R. W. G., *J. Exp. Med.*, 1930, 52, 435.

DISCUSSION

The foregoing analysis might be thought to show that colon bacilli are killed through the absorption of one quantum of ultraviolet energy within a volume not greater than a single large protein molecule.¹⁰ The most important reason for believing such an explanation improbable lies in the fact that no injurious effects have been observed in the growth of the surviving organisms which, nevertheless, must have absorbed millions of quanta apiece. These ultraviolet radiations are known to coagulate protoplasm and if one properly placed absorption

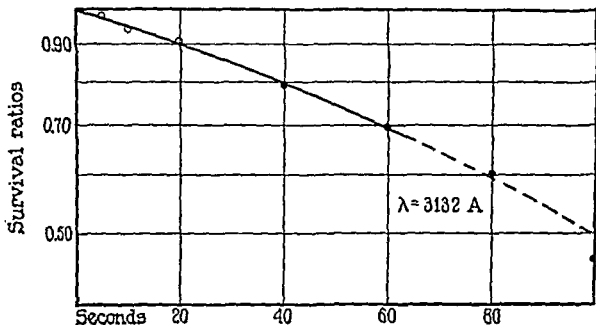


FIG. 5. Survival ratios of *B. coli* irradiated with ultraviolet light of wave length 3132 Å.

can kill, several million similar ones ought to reveal themselves by changes in the rate of growth, in colony shape or in some of the other so-called "dissociative changes" often observed among colon bacilli. No abnormalities have been seen during the present experiments but it might be profitable to carry out additional irradiations under conditions especially favorable to the perpetuation of such variants.

If the foregoing one-hit-to-kill explanation is not the correct one for these ultraviolet data, some other interpretation must be found for the semilogarithmically linear destruction of *B. coli*. Two possibilities

¹⁰ Rahn, O., *J. Gen. Physiol.*, 1929-30, 13, 179, 395.

suggest themselves. According to one, of a type often encountered in biological discussions, death is due to a process which follows the course of a monomolecular chemical reaction. It is very possible that the ultraviolet coagulation of protoplasm proceeds exponentially. Because this rate is independent of the distribution of the protoplasm it has sometimes been urged that irradiated cells should die according to the demands of a semilogarithmically linear relation. Unless death is held to be the consequence of the destruction of a single one of a cell's molecules, this conclusion is clearly fallacious. Provided such pictures based on analogies with simple chemical reaction rates are devoid of significance, as may well be the case, it would appear that the primary factor influencing the rate of bacterial death by ultraviolet light is to be found in the relative sensitivities of the bacteria themselves. Experiments have not yet been made which show whether or not this is true, but varying resistance clearly determines the rate of destruction of bacteria by heat and by certain chemical agents.¹¹

The killing experiments with bacteria thus furnish a striking example of some of the pitfalls which attend efforts to give detailed interpretations of biological reactions even when quantitative data are at hand. The rate of death is the same whether cathode rays, X-rays or ultraviolet light be used. With cathode rays the simple statistical explanation of this relation based on probability theory must quite certainly present a correct picture of the phenomena since at a sufficiently high voltage every absorbed quantum will be lethal. With X-rays, current physical ideas of the processes consequent on absorption make this type of interpretation a highly probable one, especially with the harder rays. In the ultraviolet region, however, it is likely that exactly the same quantitative biological results have a totally different explanation and one which resides not so much in the mode of action of the killing agent as in biological characteristics inherent in the organisms themselves. So little is now known of the internal constitution of bacteria that discussions of whether or not death results from the decomposition of one molecule or from some other cause involve speculations which are at the moment incapable of verification.

¹¹ Reichenbach, H., *Z. Hyg.*, 1911, 69, 171.

The writer is indebted to James R. Lucas and to C. G. Porskieves for help in carrying out these experiments.

SUMMARY

The survival ratios of colon bacilli subjected to several monochromatic ultraviolet radiations follow semilogarithmic straight lines. For each wave length approximate observations have been made of the energy involved in cell destruction. This energy varies somewhat with frequency in the ultraviolet region; it is furthermore nearly one hundred times as great as the amount of X-ray energy required to bring about the same killing.

Preliminary experiments show no measurable difference either in rate of killing or in lethal energy between *B. coli* and *B. actrycke*. Parallel results have already been obtained with X-rays and electrons.

The data from colon bacilli are interpreted in terms of the assumptions employed for X-rays. They indicate that though bacterial death should result from a single quantum absorption, millions more such absorptions seemingly are without injurious effect on cell growth and multiplication. The "sensitive volume" within which, according to this picture, the lethal quantum must be stopped proves to be about the same as that of a single protein molecule. If this is the correct description of the phenomena of ultraviolet killing, it seems strange that the millions of non-deadly quanta absorbed per bacillus should not show themselves by altered growth rates or in other ways. That they apparently do not suggests the inapplicability of the statistical picture. The death rate under this kind of radiation then would be primarily an expression of the relative sensitivities of the bacterial population. Additional experiments are required to determine this question.

THE HEAT INACTIVATION OF ANTISTAPHYLOCOCCUS BACTERIOPHAGE

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Early work on bacteriophage included several studies of the effect of heat on the lytic principle. For example, d'Herelle (1) found Shiga bacteriophage survived exposure to 65°C. for $\frac{1}{2}$ hour. Kabeshima (2) working with the same phage observed activity after $\frac{1}{2}$ hour at 70°C. and set the inactivation temperature at 75°C., using a like period of exposure. Others, particularly Weinberg and Aznar (3) and Tchang Kouo Ngen and Wagemans (4) reported that 80°C. was required for complete destruction of phage. Burnet and McKie (5) diluted phage suspensions with salts of sodium, potassium, and ammonium and found that such preparations were very easily inactivated by moderate heat (60°C.). Small amounts of calcium, magnesium or barium inhibited this effect. d'Herelle (6) summarizes his experience as follows: ". . . I have never yet found a bacteriophage, even of maximum virulence, which was not rendered totally avirulent (or destroyed?) at a temperature in the neighborhood of 75°C. nor have I found one which was rendered avirulent at temperatures below 65°C."

The concept of rate of inactivation as an important factor contributing to the experimental results appears to have been neglected except for contributions by Tomaselli (7) and Nanavutty (8). Tomaselli exposed a Shiga lysate to various temperatures and estimated the active fraction surviving after 10, 20, and 30 minutes. His data are unique in indicating that the degree of inactivation at any temperature is a function of temperature alone, being quite independent of the period of exposure. Nanavutty (8) plotted "survivor" curves for Shiga lysates suspended in various menstrua and exposed to different temperatures. He found the curves of logarithms of surviving phage particles plotted against time to be constantly concave,

tending to become asymptotic with the abscissa at low concentrations. On this basis Nanavutty concluded that bacteriophage consists of living units possessing varying susceptibilities to heat.

In the writer's opinion Tomaselli's data are inadequate for the forming of any conclusions and Nanavutty's data cannot be utilized for ascertaining the characteristics of the inactivation curve because they depend upon two definitely inaccurate methods for the quantitative detection of bacteriophage, namely, the method of serial dilution and the plaque count (9), even though Nanavutty has tried to avoid some of the sources of error by running multiple samples. Certain of the errors inherent in the serial dilution technique are considered in a recent paper (10).

Since the way in which inactivation occurs may have considerable bearing on the question of the nature of phage it was decided to study the process under carefully controlled conditions. The data in the present paper are based upon a method for the quantitative detection of phage, utilizing an arbitrary activity unit and found in the course of over 200 routine titrations to possess an accuracy of ± 2 per cent (9, 11).

Methods

An antistaphylococcus phage and a single strain of *S. aureus* described in previous papers (9, 11-14), were used. Sufficient broth (veal infusion, 1 per cent peptone, 0.5 per cent NaCl, pH 7.6) was prepared in one lot to last throughout the work. For each experiment 20 ml. of unfiltered phage containing 1×10^{10} activity units per ml. was placed in a sterile thin-walled flask. The latter was fitted with a rubber stopper bearing an accurate thermometer adjusted so that the bulb projected into the phage. The flask was immersed in boiling water and the contents quickly brought up to the temperature at which inactivation was to be followed. It was then placed in a mechanical rocker mounted in a water bath maintained at the desired temperature. Samples were withdrawn into chilled test tubes at frequent intervals and the tubes were immediately placed in ice water. Phage was determined by the author's quantitative method (9, 11). The control for each experiment consisted of a sample of the same phage shaken at room temperature.

Experimental Results

The results are shown graphically in Figs. 1-4, in which logs of residual phage units are plotted against time of sampling. Clearly, heat inactivation of phage suspended in broth proceeds logarithmically with time. In all cases the reaction was followed until there was left but a small fraction of the original active material (a residuum of from 0.001 per cent-10 per cent). This range of concentrations is adequate for establishing the characteristics of the inactivation curve.

TABLE I

Values of Velocity Constants at Various Temperatures for Heat Inactivation of Phage Suspended in Broth

Temperature	Average value of k calculated from $k = \frac{2.303}{t} \log \frac{P_0}{P_0 - P_i}$
<i>Degrees C.</i>	
51	0.00307
55	0.0207
57	0.0630
58	0.0960
59	0.1590
60	0.2350
61	0.3444
62	0.5750

Since the heat inactivation of phage at various temperatures proceeds logarithmically with time the reaction conforms to the general mass law and may be expressed as:

$$dP/dt = k (P_0 - P_i)$$

where P = Phage

P_0 = Initial P per ml.

and P_i = P /ml. inactivated in time t .

The integral form of this equation may be written: $k = \frac{2.303}{t} \log \frac{P_0}{P_0 - P_i}$ and average values of the velocity constant for each temperature may be calculated.

The solid lines in the figures represent the course of the reaction as calculated by this equation, while the points represent the experi-

mental values. The reaction evidently follows the logarithmic curve quite accurately.

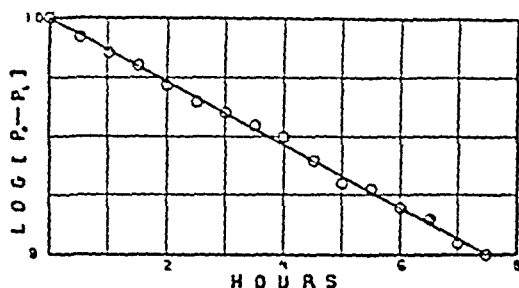


FIG. 1

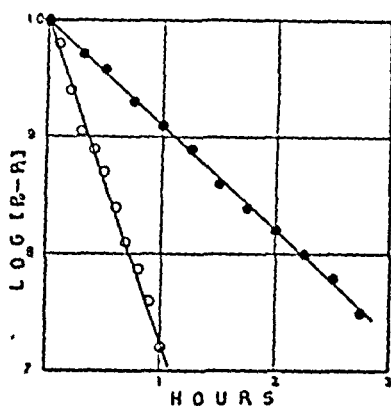


FIG. 2

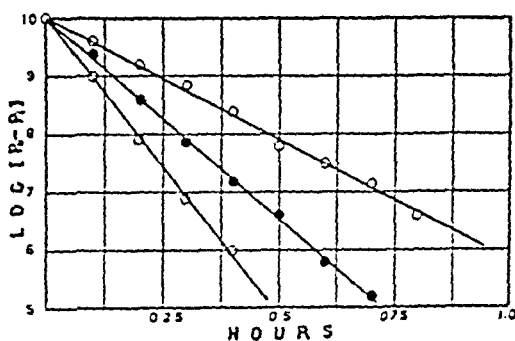


FIG. 3

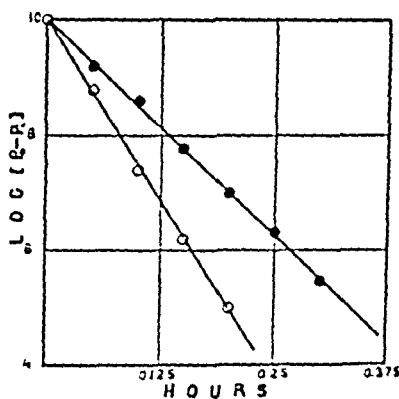


FIG. 4

FIGS. 1 to 4. Heat inactivation of antistaphylococcus phage in broth. Logarithms of residual phage units/ml. plotted against time of exposure at various temperatures. Fig. 1, 51°C.; Fig. 2, 55° and 57°C.; Fig. 3, 58°, 59°, and 60°C.; Fig. 4, 61° and 62°C.

The v'ant Hoff-Arrhenius equation concerning the relationship between rate of reaction and the absolute temperature states that:

$$k_2 = k_1 e^{\mu/2 \left(\frac{T_2 - T_1}{T_1 T_2} \right)}$$

where k_2 = Velocity constant at T_2

k_1 = Velocity constant at T_1

T_2 = Second absolute temperature

T_1 = First absolute temperature

and μ = The critical thermal increment.

As a graphic test of the constancy of μ over any given temperature range, it is desirable to plot values of $\log k$ against the reciprocals of the absolute temperatures. If μ is a constant the plot will be a straight line of slope $-\mu/4.606$. Fig. 5 is such a plot and demonstrates the constancy of the critical thermal increment over the temperature range 51°–62°C. The value of μ is found to be 101,000.

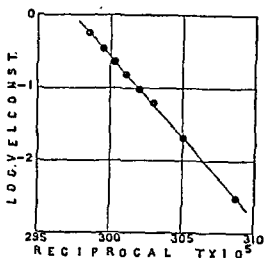


FIG. 5. Plot of logarithms of velocity constants for heat inactivation of phage at various temperatures against the reciprocals of the absolute temperatures.

DISCUSSION

The heat inactivation of antistaphylococcus bacteriophage suspended in broth of pH 7.6 at temperatures ranging between 51°C. and 62°C. proceeds strictly in accordance with the equation for a monomolecular reaction. Consequently the experimental data furnish no evidence that ordinary phage lysates consist of particles possessing varying degrees of resistance to heat, as Nanavutty found. Nanavutty felt that his experiments strongly suggested the living nature of phage. The present data cannot be so interpreted. For, while it is true that many unicellular organisms follow a logarithmic order of death upon exposure to a variety of lethal agents, the inactivation of certain enzymes is similarly an exponential process.

The average value of μ , the critical thermal increment, for heat inactivation over the temperature range studied was found to be 101,000. It is therefore of the same order of magnitude as the values of μ determined for such processes as the heat denaturation of egg white (15), the spontaneous destruction of rennet, invertase, vibrio-

lysin, etc., and for the course of death among some bacterial populations (16).

Very high critical thermal increments for destructive (denaturation) processes apparently occur only in protein compounds. The high value of μ for heat inactivation of phage would indicate then that protein denaturation is very likely the chief reaction involved in the process. However it does not necessarily signify that phage is a protein for it has been shown that the actual lytic material is carried on vehicular particles from which it can be separated under certain conditions (17, 18). The high value of μ may merely mean that the carrier particles possess a high temperature coefficient for heat denaturation and that the change produced in them results in inactivation of attached lytic substance.

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INTERMITTENT STIMULATION BY LIGHT

I. THE VALIDITY OF TALBOT'S LAW FOR MYA

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I

Talbot's Law

When the illumination of a visual field is interrupted with sufficiently high frequency, it appears to the human eye as continuous. Talbot (1834) first pointed out that the brightness of such a field now corresponds to the original illumination multiplied by the fraction which the actual duration of illumination is of the total duration of a complete cycle of illumination and darkness. Thus, a reduction in the time of action of the light is equivalent *visually* to a corresponding reduction in its intensity.

Talbot, himself, records no measurements in terms of which the validity of his law may be judged, though his paper clearly indicates that he made such measurements. These were first given by Plateau (1835) who compared the brightness of rotating white discs containing black sectors with the brightness of the same white cardboard at different distances from a source of light. At equal brightness, he found that the square of the distance of the rotating disc from the light is to the square of the distance of the white paper as the angle of the white sector is to 360°. The measurements are limited, but adequate. Later, Helmholtz (1865) described several additional ways of demonstrating Talbot's law, and appears to have tried them; but he gives no measurements.

The first to doubt the validity of Talbot's law was Fick (1863) who, in thinking about the matter, laid down a surprisingly adequate mathematical basis for the whole process. Fick recognized that at the stationary state, when an intermittent illumination produces a con-

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tinuous impression, the velocities of the rise and of the decay of the excitatory process (An—und Abklingen) must be equal regardless of the relative durations of light and dark exposures. But that this should result in so simple a mathematical relation as Talbot's law seemed impossible to Fick in the light of what was to him the obviously complicated nature of the light and dark processes themselves.

Fick's own measurements, using essentially Plateau's technic, convinced him that Talbot's law is not valid. However, as Aubert (1865) clearly showed, Fick's own measurements do not support his contention. They are indeed quite adequate to verify the law within the rather large experimental error incident to his method of measurement.

It may be added that Fick's ideas of the complexity of the retinal processes, though justified on general principles, have not been borne out by later developments. Modern work on the physiology of vision, notably on light and dark adaptation (Hecht, 1931; Kohlrausch, 1931), has shown these processes to be much simpler than Fick could have anticipated.

The position of Talbot's law remained where Fick and Aubert left it until Kleiner reinvestigated the whole matter in 1878, using Zöllner's polarization photometer. His data show quite clearly that within a photometric error of a few per cent Talbot's law holds for the sector openings he measured, which ranged from 180° to about 1.5° . These results have been confirmed by Wiedemann and Messerschmidt (1888) using only a few sectors, by Ferry (1893), and by Lummer and Brodhun (1896). Lummer and Brodhun state that according to their extensive measurements, made at the Reichsanstalt, Talbot's law holds to within 0.5 per cent, but neither they nor Ferry record any of their data.

Apparently the doubt raised by Fick seems still to have persisted, because in 1906 Hyde deemed it necessary to study the situation all over again in full detail. Hyde's work, done at the Bureau of Standards, is a model of precision. He investigated sector openings between 10° and 288° and found the results to be in agreement with expectation from Talbot's law within a photometric error of 0.5 per cent. For the human eye, therefore, there can no longer be any doubt of the validity of Talbot's law.

Certain points, however, remain to be noted. Though the deviations from Talbot's law found by Hyde are below 0.5 per cent, they are not haphazard, but systematic. This is true also of Kleiner's measurements, and of Fick's before him. Similarly, Grünbaum (1898) noted rather large deviations (12 to 14 per cent) at extremely high intensities, though he found Talbot's law to hold within about 1 per cent at ordinary illuminations. These deviations are undoubtedly significant, and may serve to give second order information about the visual process.

A completely different matter is the work of Parker and Patten (1912) which has been taken as contradicting Talbot's law. Parker and Patten found that two lights—one continuous and one intermittent—which had been made equal in brightness visually, gave different "energy" values when measured by a radiomicrometer, the intermittent light giving a reading about 5 per cent greater than the continuous.

In explanation of Parker and Patten's findings, it should be emphasized, as indeed Talbot himself emphasized it nearly one hundred years ago, that Talbot's law is a *physiological* generalization, not a physical one. A physical arrangement which will follow Talbot's law must be constructed in certain essentials like the eye and brain. A radiomicrometer is not such an arrangement because its response to light and its recovery are both slow in comparison to the frequency of flicker required by the eye for fusion. A photoelectric cell, which can follow faithfully the rapid alternation of light and dark, is a more likely instrument for recording the energy content of a beam of intermittent light.

Of Talbot's law for animals other than man, enough is known to make its general validity probable. Ewald (1913-14) found that the eye of *Daphnia* could not discriminate between continuous light whose intensity was reduced with a diaphragm and intermittent light reduced by a sector of corresponding magnitude. The same is stated by Patten (1914) to be true for the orientation to light of the blowfly larva. Ewald investigated only three discs, transmitting $\frac{1}{2}$, $\frac{1}{4}$, and $\frac{1}{10}$ of the light, whereas Patten apparently used only one sector disc of unspecified aperture. Later measurements by Loeb and Northrop (1917) and by Northrop and Loeb (1923) showed that

barnacle larvae and *Limulus* follow Talbot's law in their phototropic behavior to light. In both cases Loeb and Northrop used only two sector openings: 90° and 144° with the barnacle larvae, and 90° and 180° with *Limulus*. Patten as well as Loeb and Northrop refer to their results as proving the Bunsen-Roscoe law; however, it is apparent, as Ewald (1913) has already pointed out, that they mean Talbot's law.

With *Daphnia*, Ewald had found that Talbot's law held only when the alternation of light and dark was greater than thirty times per second. At frequencies below thirty per second, the intermittent light became less or more effective depending on the particular reflex chosen for measurement. Apparently thirty cycles per second is the critical fusion frequency for *Daphnia* for the illuminations used. Below the critical fusion point other phenomena probably come in.

This is borne out by the work of Dolley (1923) and of Mast and Dolley (1924), who found with several species of insects that the effectiveness of interrupted light at low frequencies of interruption varies with the rate of flicker. At some middle rate the animals are stimulated more than at a higher or a lower flicker rate. These phenomena occur at rather low frequencies of interruption—between 12 and 20 per second, and are most likely below the point of fusion. A similar situation seems to occur in human vision when, as first found by Brücke (1864) and later confirmed by Exner (1870), an interrupted light *while it still appears flickering*, seems brighter than when it has fused. This phenomenon is therefore not strictly related to Talbot's law which deals only with illuminations interrupted with frequencies sufficiently above the fusion point to give a continuous visual effect.

II

Physiological Significance of Talbot's Law

Talbot realized that the law which has come to bear his name is a physiological generalization, and depends on the properties of the visual mechanism. Since little knowledge of retinal physiology was then available, he could not deduce such a relationship, and therefore stated that "its proof can rest upon experiment alone." He added that "by that it appears to be most satisfactorily established," a

statement in which we can concur as a result of the work just enumerated.

For us the validity of Talbot's law is important, because it can serve to define those kinetic aspects of the visual mechanism which are responsible for its validity. This was recognized by Fick (1863), who, it will be recalled, was so certain of the complexity of the visual process that he denied *a priori* the validity of Talbot's law. Exner (1870), being deterred by no such limitations, tried to derive from Talbot's law the curves for the rise and fall of the sensation produced by light. Because of the arbitrary nature of the details of these curves, they have been of only limited use. They are of importance, however, in indicating that the significance of Talbot's law as a tool for understanding the retinal processes was fully appreciated fifty years ago.

Our approach to Talbot's law is a different one from Fick's and Exner's. We shall try to do what Talbot, for complete lack of data, could not do, namely, to start with the known properties of the photosensory process and to derive Talbot's law from them.

In the course of the last few years the nature of the processes concerned with photoreception has received a certain clarification by the construction of tentative hypothetical systems dealing with the physical chemistry underlying the photosensory process (Hecht, 1931). If these hypothetical systems have more than *ad hoc* value, it should be possible to derive from them expressions for the effects of intermittent light, and in particular for the validity and limitations of Talbot's law.

The present paper records our work with the clam, *Mya arenaria*. We propose first to show how Talbot's law may be derived on purely theoretical grounds from the equations which have been previously found to describe measurements of different aspects of the photosensory behavior of *Mya*; and second, to present the details and measurements of our experiments made to test in two ways the validity of Talbot's law for *Mya*.

III

Theoretical Derivation of Talbot's Law

Many previous measurements have shown that the primary process in the photoreception of *Mya* behaves like a reversible photochemical reaction. It is supposed that a photosensitive substance S is decom-

posed by the light, and that the major decomposition products P and A by themselves or with the help of another substance or source of

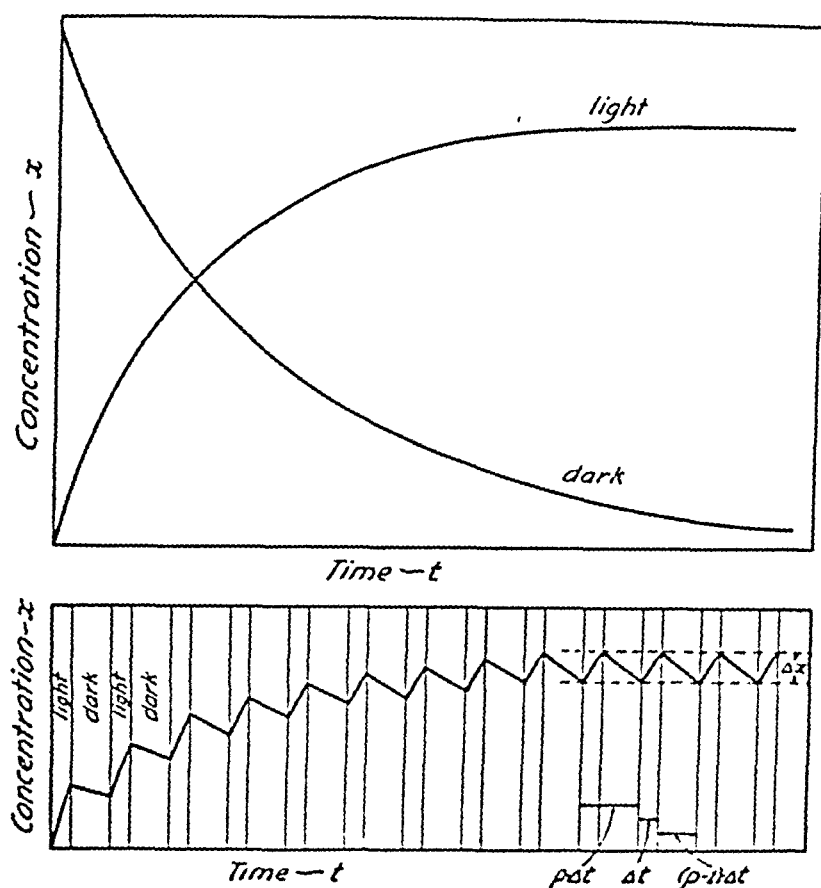


FIG. 1. Diagrammatic derivation of Talbot's law. The upper two curves represent the course of light adaptation and of dark adaptation. For *Mya*, dark adaptation is in reality many times slower than light adaptation. The two processes are made roughly alike in speed here purely for diagrammatic purposes, since the actual values do not enter into the derivation of Talbot's law as given in equations (1) to (9). In the lower part, the alternation of light and dark periods is such that the light period is given by the small time Δt , the total cycle of light and dark periods by $p\Delta t$, and the dark period by $(p-1)\Delta t$. The jagged line is constructed from the two upper curves of light and dark adaptation by drawing for each light and dark period that portion of the upper curve which corresponds to the particular value of the ordinate x which the jagged line reaches at each light and dark period. The fluctuation in concentration, Δx , at the final period of the stationary state is to be considered quite small in relation to the concentration x .

energy reunite to form S by means of an ordinary dark reaction. The reaction as a whole is a reversible one, possibly completely, but more likely only pseudoreversible.

Consider then the kinetics of such a photochemical reaction $S \rightleftharpoons P + A$. Let a be the initial concentration of S in the dark. The system is exposed to light whose intensity is I . Let x be the concentration of S which has been transformed to form a corresponding concentration of P and A . The amount of S remaining is $a - x$. The velocity of decomposition of S is then

$$\frac{dx}{dt} = k_1 I(a - x) - k_2 x^2 \quad (1)$$

where k_2 is the velocity constant of the dark reaction and k_1 is the velocity constant of the photochemical reaction proper and already includes the absorption coefficient of the sensitive material S (Hecht, 1924). That the "dark" effect must be included in a description of the light effect was apparently first recognized by Exner (1870). The course of this light reaction is shown in Fig. 1. If the light is permitted to shine indefinitely, it will induce a stationary state in which the concentrations of S , P , and A remain constant since $dx/dt = 0$. Equation (1) becomes then

$$\frac{k_1}{k_2} I = \frac{x^2}{a - x} \quad (2)$$

the familiar expression for the stationary state.

Corresponding to any value of I in equation (2) there is a given concentration of x . Thus a reduction of the intensity to I/p by some purely physical means such as a diaphragm or a filter will yield

$$\frac{k_1}{k_2} \cdot \frac{I}{p} = \frac{x_p^2}{a - x_p} \quad (3)$$

which describes a specific value of x in equation (2) as determined by I/p .

What effect will there be on this photochemical system when we reduce not the intensity, but the time during which the intensity acts, by means of a sector disc? The system is now subjected to alternating periods of illumination by intensity I and of darkness. Fig. 1 will

help in describing what happens. During the light period the velocity of the process will follow equation (1) and the concentration x will increase; while during the dark period, only the "dark" reaction, whose velocity is given by

$$\frac{dx}{dt} = k_2 x^2 \quad (4)$$

will proceed. This is essentially a short period of dark adaptation during which x will decrease. If the alternation of light and dark periods is maintained, a pseudostationary state is reached in which the dark recovery becomes equal to the light effect, and x fluctuates equally above and below a mean value. With rapid alternation of light and dark periods the rise and fall in concentration x becomes small until, when flicker disappears, the change in concentration of x , which we may now call Δx , becomes too small to be effective in producing a change in the sensation of brightness.

Fig. 1 makes the matter clear. The vertical distance is Δx and is to be considered as very small. Let the light period be Δt , a short interval of time, and let the dark period be $(p-1) \Delta t$. The total time of a cycle of light and dark exposures is $p \Delta t$ and $1/p$ is the fraction of the total exposure time occupied by the light exposure alone. During the short time Δt the velocity of the light reaction will be

$$\frac{\Delta x}{\Delta t} = k_1 I(a - x) - k_2 x^2 \quad (5)$$

whereas the velocity of the dark reaction during the short time $(p-1) \Delta t$ will be

$$\frac{\Delta x}{(p-1) \Delta t} = k_2 x^2 \quad (6)$$

since as much recovery must take place during the dark as decomposition in the light. Equation (6) may be transformed into

$$\frac{\Delta x}{\Delta t} = (p-1) k_2 x^2 \quad (7)$$

which can now be equated to (5). This relation is

$$k_1 I(a - x) - k_2 x^2 = (p-1) k_2 x^2 \quad (8)$$

which on combination of terms and solving, becomes

$$\frac{k_1}{k_2} \cdot \frac{I}{p} = \frac{x^2}{a - x} \quad (9)$$

an equation identical with (3). In other words, a reduction to a given fraction $1/p$ in the time of action of a light by alternating it rapidly with periods of darkness is exactly equivalent to a reduction to the same fraction of the intensity of a continuously acting light. This is Talbot's law, which has now been derived from previous work with *Mya* without the addition of any new information or assumptions.

A word of caution may be added here with regard to the meaning of the equations and symbols used in this derivation and in previous work with *Mya*, and indeed with the human eye as well. We have referred to substances *S*, *P*, *A*, and to their concentrations *a* and *x*. If it pleases any one better, these may be considered as the various levels or intensities of the rise and fall of the "excitatory" process; the quantitative relationships will maintain their validity, though no picture will be available for the mechanism.

Indeed one need refer neither to a photochemical reaction nor to an "excitatory" process, but to the original measurements of light and dark adaptation. It is found by direct measurement that dark adaptation follows a certain course, and may be described quite empirically by an expression like equation (4) in which *x* refers to measurements of reaction time, or of intensity thresholds. Similarly the course of light adaptation may be measured, but not so directly. Its kinetics may be described in terms of equation (1) in which here again *x* will be some function of the measured values of the reaction time or of the threshold intensities. The remaining algebraic manipulations follow exactly as before, and the result is that in terms of measurements of light and dark adaptation one may predict the validity of Talbot's law. We ourselves prefer to interpret the equations in terms of a photochemical system, purely for the convenience in thinking concretely about the receptor process.

A similar situation concerns the precise meaning of the value Δx . We have described it here as that fluctuation in concentration *x* which becomes too small to be effective in producing a sensory change in intensity reception. It is not possible at present to say whether this

corresponds to a non-effective fluctuation in the frequency of impulses which pass over the nerve fiber from the sense cell, or whether it corresponds to that value of the intensity fluctuation which will fail to elicit a change in the number of elements functional in the sensory layer. It may mean any of these three aspects of the matter—concentration, frequency, or number—or all of them, or perhaps some other, still unknown aspect of the sensory process. The derivation of Talbot's law as here given remains unaffected.

IV

Nature of Measurements

Having deduced Talbot's law for the photosensory behavior of *Mya*, we then devised two series of experiments for testing this deduction. The first series was made with seven discs having openings ranging from 2 per cent to 100 per cent of the disc. For each disc the intensity of light was computed which, when combined with the rapidly rotating disc, will furnish a given amount of energy. The light from each combination now appears the same to the human eye, and is indistinguishable from a continuous light delivering the same energy over the same period of time. If Talbot's law holds for *Mya*, the photosensory behavior of an animal exposed to such lights should always be the same regardless of the combination of light and sector opening producing the illumination, and should also be indistinguishable from its behavior to a continuous light of corresponding energy content. This was found to be true.

In the second series of measurements the same discs were used with a constant outside intensity so as to produce a series of beams differing in energy content. Each beam of interrupted light was matched in energy content by a beam of continuous light, and the photosensory effect of the two groups of lights were compared. The results showed that, judged by its photosensory behavior, *Mya* could not distinguish between intermittent light and continuous light of the same energy content.

The particular aspect of the photosensory behavior of *Mya* which we used in these experiments is the capacity of *Mya* for intensity discrimination. It has been shown (Hecht, 1923) that the clam, when exposed to sustained illumination, at first responds by contracting its

siphons, and then rapidly comes into sensory equilibrium with the light. This is of course the process of light adaptation. To produce another contraction of *Mya*'s siphons, the light must be suddenly augmented. The reaction time to this added illumination depends on the intensity of the adapting light and on the intensity of the added (or stimulating) light. With a constant stimulating intensity the reaction time increases in a specific way as the adapting intensity increases. Our purpose then was to compare the reaction time of *Mya* to a stimulating light of constant intensity after it had been adapted for some time to an illumination of given energy content furnished on the one hand by intermittent light secured by a variety of sectors and on the other hand by continuous light.

V

Apparatus and Calibrations

The measurements were made at the Marine Biological Laboratory, Woods Hole, Massachusetts, during the summer of 1928. The experimental arrangements may be made out by means of Fig. 2. We used two dark rooms separated by a wall, and communicating through an opening in the wall. In dark room A were placed the adapting light, its metal housing, the motor, the sector discs, as well as various screens for keeping all but the direct light of the lamp from entering the second dark room B through the opening in the wall. The second dark room contained a long, solidly mounted, black table on which there was a board about 30 cm. square which could be moved along the table and placed in any position on it. On this black painted board there were marked cross-lines as shown in Fig. 2 which indicated the exact position and direction for the placing of the siphon of an animal in a rectangular glass dish. About 20 cm. away and in the position indicated in Fig. 2 there was mounted on the board a 100 watt, concentrated-filament lamp in a light-tight container with an opening 3 cm. square facing the animal. This was the stimulating light. A shutter in front of the opening served to expose the animal to the stimulating light at the proper time and with proper precision, the light having been first turned on by a switch.

The sector discs were made of aluminum 2 mm. thick and 200 mm. in diameter. Each disc had two sector openings 180° apart. The

discs were painted dead black. Each disc could in its turn be mounted directly on the shaft of the motor which was rotated at a speed of 4000 R. P. M. Thus during the experiments there were approximately 130 cycles of light and dark flashes per second, a rate well above any recorded value of the critical frequency for fusion at the highest brightness for the human eye.

In view of the complete validity of Talbot's law for the eye we calibrated the sector openings photometrically. The diffusely reflecting plate of a Macbeth illuminometer was placed in the position of the animal, and at 45° to the direction of the beam of light. The

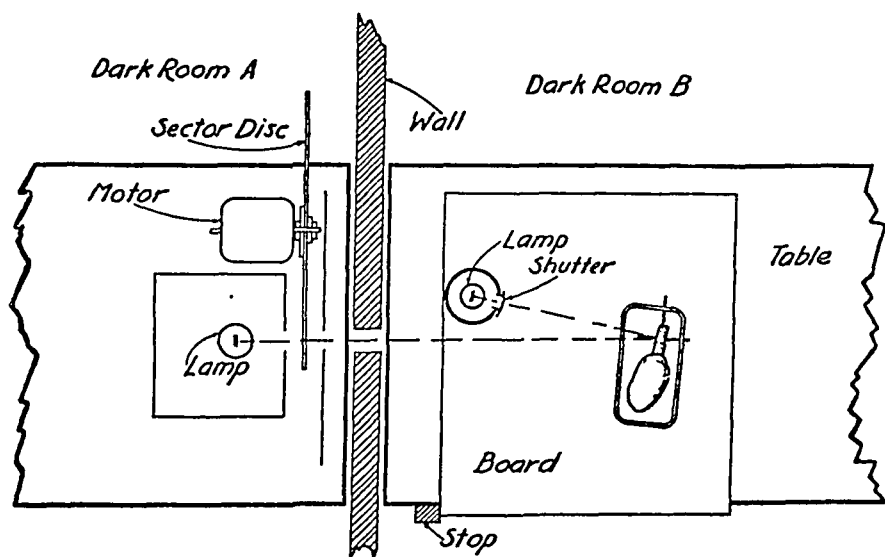


FIG. 2. Arrangement of apparatus.

light was a 400 watt, concentrated-filament lamp, and was run on 110 volts direct current; the current was kept constant with a rheostat and an ammeter. The Macbeth illuminometer itself was securely clamped at right angles to the beam of light so as to view the center of the illuminated diffusing plate. With everything clamped in position we made three separate series of calibrations of the brightness of the plate in the direct light of the adapting lamp, and with the various sector discs in operation. We each made ten readings of the brightness for each sector disc. The results of the three series agreed satisfactorily; we have therefore averaged them. The transmissions of the sector discs given in Table I are thus the averages of 60 readings each.

In order to make up combinations of sector disc and intensity so as to yield a given value of energy with which to adapt *Mya*, we needed a variable intensity. This we wished to secure by placing the board, on which rested the animal and the stimulating light, at different

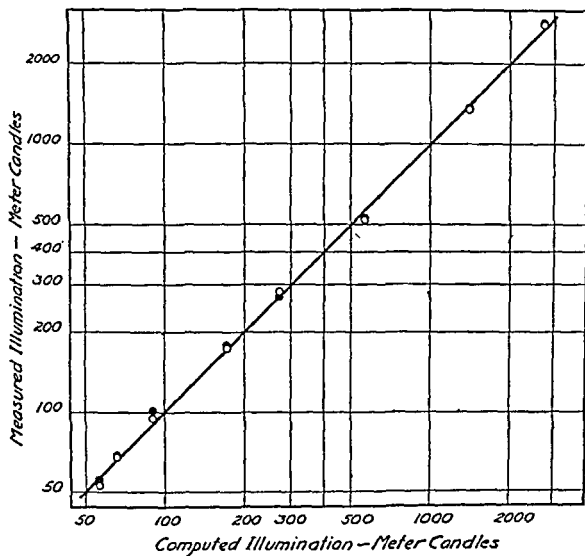


FIG. 3. Relation between illumination and distance from the adapting lamp. The ordinates are the measured values for *H* (open circles) and for *IV* (solid circles) whereas the abscissas are computed from the inverse square of the distance. The coordinates are logarithmic, the better to show up the errors at low intensities.

distances from the adapting light in the next room. Using the same Macbeth illuminometer, the same 400 watt lamp, and the same diffusing plate in the same position on the board as before, we determined the brightness of the diffusing plate at different distances from the lamp.

In making these measurements we had no idea other than to construct a calibration curve relating distance from the lamp along the table and resulting intensity of illumination. In computing the measurements we found that under the circumstances (a concentrated filament lamp, and complete screening of extraneous and reflected light) the data followed the inverse square law well within the limits of our photometric error. This is not unexpected in view of the thorough test to which Hyde (1906) has subjected the inverse square law for other than point sources. However, since the statement is so frequently made that the inverse square law does not apply, we give our measurements in Fig. 3. The figure shows a comparison between our values and those computed in terms of the inverse square ratio. Each point is the average of ten readings. The data are plotted on a logarithmic grid the better to show the errors at low illuminations.

VI

Measurements with Constant Adaptation Secured in a Variety of Ways

For the first series of measurements we used an adapting intensity of 55 meter candles. From our calibrations we computed for each sector disc the distance from the 400 watt lamp at which the clam must be placed on the table in order for the disc and the light to furnish this illumination. The combination of distances and sectors are to be found in Table I. This set-up now enables us to place an animal in such a position with each sector that the illumination on it is constant even though secured by different intensities and different ratios of light and dark exposures. To accomplish such a procedure easily and securely we fixed a series of wooden blocks on the edge of the table, so arranged that the movable board containing the stimulating light and the animal fitted against them by means of an overhang of the board which slid along the edge of the table. In this way the board could be repeatedly placed accurately in any position in a few seconds.

The procedure in this series of measurements was of the following nature. The board was placed in a given position, the corresponding sector disc was attached to the motor and the motor started. The adapting light was turned on and an animal placed in the proper spot on the board. The animal remained exposed to this adapting illu-

mination for 5 minutes. At 5 minute intervals its reaction time was measured three times to the stimulating light which had an intensity of 2250 meter candles. The animal was then placed in the dark for half an hour. After this it was exposed to the same adapting illumination as before, but secured by a different combination of distance and sector disc. It was given 5 minutes adaptation and three more readings of its reaction time to the same stimulating light as before were made.

TABLE I

Reaction time of ten animals to a stimulating light of 2250 meter candles after adaptation to 55 meter candles obtained either by continuous illumination or by intermittent illumination furnished by seven different combinations of rotating sector openings and distances from the light.

Sector disc	Calibrated transmission of sector	Distance of animal from lamp	Relative reaction time										
			Animal No.										Average
			I-1	I-3	I-5	I-8	I-9	I-10	I-11	I-12	I-13	I-14	
	per cent	cm.											
I	2.19	51.5	1.00	1.06	1.01	0.96	1.00	0.98	0.96	1.01	1.01	0.94	1.00
II	4.38	72.8	1.00	0.96	1.03	1.04	0.96	1.05	1.09	1.03	0.97	1.04	1.02
III	10.69	113.7	0.98	1.00	1.01	1.06	0.91	0.91	0.96	0.98	0.93	0.98	0.97
IV	19.83	154.8	1.02	1.05	0.92	0.99	0.91	0.95	1.04	1.07	0.99	0.90	0.98
V	25.00	173.8	1.02	1.01	0.94	1.01	0.98	1.05	0.98	1.01	1.01	1.03	1.00
VI	51.80	250.2	1.00	0.96	1.03	0.99	1.00	1.00	0.98	0.98	1.05	0.97	1.00
VII	76.54	304.2	1.00	1.03	1.03	1.01	1.05	1.02	1.01	0.98	0.99	1.03	1.02
No disc	100.00	347.7	1.00	0.92	1.05	0.94	1.10	1.05	1.01	0.98	1.05	1.00	1.01
Actual average reaction time in seconds...			1.30	1.98	1.88	1.35	1.43	1.50	1.56	1.19	1.75	1.67	

Our experience has shown us that 5 minutes light adaptation is adequate. The adapting light was kept on, of course, all the time during and between the measurements of the reaction time, so that when the last reaction time was measured for each combination of disc and distance the animal had been adapting for 15 minutes. No difference in reaction time was found between the first and the last readings.

Continuing in this way, the seven combinations of distance and sector disc were run through with an individual animal. Somewhere in the series we also adapted the animal to continuous light of the

same brightness and measured the reaction time three times at 5 minute intervals. To make these eight sets of measurements with a single animal required a day.

We measured ten animals in this way. The data secured are given in Table I. For simplicity in presenting the data we give at the bottom of the table for each animal its average reaction time for all the measurements; that is the average of twenty-four readings. This value is then put at 1.00, and for each combination of disc and intensity the corresponding reaction time (average of three readings) is given as a fraction of the average value. In this way all the animals are immediately comparable.

Since the reaction time depends upon the adapting intensity, then, if Talbot's law holds, the reaction time to these eight adapting combinations of sector and distance should be identical. If not, they should show some systematic variation relating them to the size of the sector opening or to the impinging intensity. Table I shows that for each animal the reaction time varies in no specific way; and that its general variation is of the order expected in these measurements.

This can be seen by comparing the measurements for a given animal in a vertical column with the measurements for the different animals in a horizontal column. The averages of the ten animals for each combination of sector and intensity never differ more than 3 per cent from the mean of all the readings, and most of them differ by less. The average difference from the mean is 1.2 per cent, which is well below our photometric error using the present apparatus. Therefore, this series of experiments indicates that for the clam Talbot's law is valid, and that the clam does not distinguish between a continuous and an intermittent illumination of the same brightness.

VII

Measurements with Different Adaptation Intensities

After completing the measurements just recorded, we considered it too precarious to rest a conclusion on a single intensity, even though it was secured in eight different ways. Our previous work (Hecht, 1923) has shown that the relation between adapting intensity and reaction time is sigmoid, and possesses a fairly flat portion in which the

reaction time changes very slowly with the adapting intensity. It seemed possible that the particular intensity which we investigated might lie in this region,—in which case measurements of the reaction time are not delicate enough to show up slight differences in intensity. This, in fact, is precisely the case, as Fig. 4 shows. However, the relation between adapting intensity and the reaction time to a constant stimulating light also has two very steep portions. In these portions

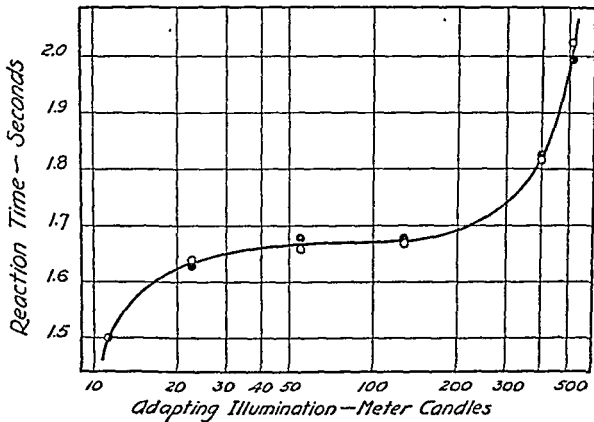


FIG. 4. Relation between adapting intensity and reaction time to a constant stimulating light. The open circles are for adaptation to continuous light, whereas the solid circles are for adaptation to intermittent light. The two produce the same results.

the reaction time is a very delicate measure of the intensity. We therefore decided to investigate the relationship over a sufficiently wide range of adapting intensities to bring out the phenomenon as a whole, including the two very steep, and for our purposes, critically valid sections.

The second series of measurements was made with a variety of adapting intensities. Each adapting intensity was secured in two

ways—by continuous illumination and by intermittent illumination—and the reaction time to a constant stimulating light measured for each. We used the same discs as before, but instead of a 400 watt lamp, we used a 100 watt lamp for the adapting source; the stimulating light remained the same, and gave an illumination of 2250 meter candles.

The measurements were made as follows. For intermittent light the board was always in one position, at 51.5 cm. from the light. A sector disc was placed on the motor and the motor put in action. An

TABLE II

Reaction time of six animals to a stimulating light of 2250 meter candles after adaptation to a series of illumination intensities secured by continuous light and by intermittent light. (I = intermittent; C = continuous).

Intensity in meter candles	Reaction time in seconds													
	Animal No.												Average	
	II-3		II-4		II-5		II-6		II-7		II-8		Average	
	I	C	I	C	I	C	I	C	I	C	I	C		
11.29	1.60	1.57	1.50	1.43	1.57	1.57	1.53	1.60	1.53	1.53	1.27	1.30	1.50	1.50
22.58	1.73	1.70	1.57	1.77	1.77	1.70	1.67	1.63	1.70	1.67	1.33	1.37	1.63	1.64
55.11	1.80	1.80	1.77	1.70	1.70	1.67	1.70	1.67	1.67	1.67	1.47	1.43	1.68	1.66
128.9	1.90	1.87	1.77	1.73			1.67	1.67	1.67	1.63	1.40	1.43	1.68	1.67
394.6	1.97	2.00	1.87	1.83	1.83	1.87	1.80	1.87	1.93	1.83	1.60	1.50	1.83	1.82
515.5	2.00	2.23	1.97	2.00	2.17	2.20	1.97	1.90	2.00	2.03	1.87	1.83	2.00	2.03

animal was then exposed to the resulting illumination for 5 minutes. adaptation, and its reaction time taken three times at 5 minute intervals as before. It was then given 15 minutes in the dark. After this, the board was moved to such a distance from the light that the animal would receive the same illumination with continuous light as it had previously had with intermittent. It was given 5 minutes adaptation, and its reaction time to the same stimulating light as before was measured three times at 5 minute intervals. It was then given 15 minutes in the dark. The board was then replaced in its previous position, a new disc placed on the motor, and the animal exposed for adaptation to the light from a new disc and 100 watt

lamp for 5 minutes. Its reaction time was then measured three times as before. Continuing in this way the animal was adapted to a series of different intensities, each paired so as to be continuous or intermittent. In each case its reaction time was measured in the usual way, three times at 5 minute intervals.

For this series of measurements, we used only six discs so that with each animal there were made twelve groups of three reaction time measurements. It was possible in this way to do one animal a day.

The results are in Table II which gives the average reaction time for each of six animals to light of 2250 meter candles when the animal has been adapted to a series of five intensities produced by intermittent light and by continuous light. For the highest illumination, we measured the reaction time of the animal adapted to continuous light two times: once with the motor running as in the case of intermittent illumination but with no sector disc attached, and once with the motor quiet as was usual with continuous light. This served as a check on the adequacy of the measurements. It is apparent from comparison of the reaction time of the animals after adaptation to intermittent illumination and after adaptation to continuous illumination that there is no difference in the reaction time of the animals under the two conditions. This is brought out by the average of the six animals shown in the last two columns of the table. The largest difference happens to be between the readings made under the identical condition of adaptation to continuous illumination except that the motor was running in one series and not running in the other.

The results are shown graphically in Fig. 4, and it is apparent that the reaction time in the two cases follows exactly the same relationship to the adapting intensity regardless of whether it is an intermittent or a continuous illumination. This is particularly the case in the two rapidly changing portions of the curve, where any slight difference between the two would at once become evident.

We may then conclude from these two series of measurements that as predicted from purely theoretical considerations of its dark and light adaptation, *Mya* cannot distinguish between intermittent and continuous illumination of the same visual brightness. It is therefore shown to obey Talbot's law within the limits of error of these measurements.

SUMMARY

On the basis of previous knowledge of the photosensory behavior of *Mya* it is shown that Talbot's law for the effectiveness of stimulation by intermittent illumination should be valid. Two series of measurements are reported in which the photosensory effects of intermittent and continuous illuminations are compared. The results demonstrate the validity of Talbot's law for *Mya*.

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A SEPARATION OF THE REACTIONS IN PHOTOSYNTHESIS BY MEANS OF INTERMITTENT LIGHT

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Experiments on photosynthesis in intermittent light have been made on two occasions. Brown and Escombe, in 1905, made use of a rotating sector to study the effect of light intensity on the photosynthesis of leaves. They found three-quarters of the light from a given source could be cut out in each revolution of the sector without decreasing the rate of photosynthesis. Willstätter (1918, p. 240) explains that this was probably due to the low concentration of carbon dioxide available for the leaves. The short periods of light would be sufficient to reduce all the carbon dioxide which could reach the cells by diffusion during the dark periods.

In 1919-20 Warburg made experiments on *Chlorella* similar to those of Brown and Escombe on leaves. Instead of stating his results as amount of photosynthesis per total elapsed time, as Brown and Escombe did, he gave photosynthesis per total time during which the cells were illuminated. Since he used sectors which cut out half the incident light in each revolution, the time during which the cells were illuminated was always half of the elapsed time of an experiment.

Working with a high intensity of light and a high concentration of carbon dioxide, Warburg found that a given amount of light reduced more carbon dioxide when allowed to fall on the cells intermittently than when allowed to fall on them continuously. The improvement in the yield of the intermittent over the yield in continuous light depended on the frequency of the flashing. With a frequency of four periods per minute the improvement was 10 per cent, and with a frequency of 8000 per minute it was 100 per cent.

Warburg proposed two alternative explanations for the improvement in the yield of the intermittent light. Either the reduction

of carbon dioxide continues in the dark, or it proceeds twice as fast during the brief light flash as during the same length of time in continuous light. He considers the latter explanation more likely, and assumes that certain steps in the photosynthetic process continue in the dark until a dark equilibrium is reached. After the dark period a short flash of light would find a higher concentration of reactive substance ready for it than is available in continuous light, and would be able to effect more decomposition than an equal amount of continuous light.

The experiments described in this paper indicate, we think, that the steps in photosynthesis which proceed in the dark involve what has hitherto been known as the Blackman reaction. Probably the reduction of carbon dioxide is not completed during the photochemical part of the process. A more correct way of representing the sequence of events in intermittent light would be as follows. Two steps are involved in the reduction of carbon dioxide: a reaction in which light is absorbed, followed by a reaction not requiring light—the so called Blackman reaction. If the light intensity is high the photochemical reaction is capable of proceeding at great speed, but in continuous light it can go no faster than the Blackman reaction. We suppose that the product formed in the photochemical reaction is converted to some other substance by the Blackman reaction, and at the same time the chlorophyll is set free to take part again in the photochemical reaction. If a green cell is illuminated, we think that the photochemical reaction proceeds rapidly until an equilibrium concentration of its product is formed. After this the photochemical reaction proceeds only as fast as the Blackman reaction removes the intermediate product. If the cell is now darkened, the photochemical reaction stops at once, but the Blackman reaction continues until its raw material, the product formed by the photochemical reaction, is exhausted. After this nothing further happens until the cell is again illuminated. Higher efficiency of the light would be obtained if each light flash lasted only long enough to build up the equilibrium concentration of the intermediate product, and each dark period were long enough to allow the Blackman reaction time to use up all the intermediate product present at the moment the light period ended. In Warburg's flicker experiments the light and dark periods were always

of equal length. He found that the amount of work done by the light could be increased by shortening both the light and the dark periods. This indicates that his light periods were too long for maximum efficiency. In the latter part of each light period the photochemical reaction must have been brought down to near the speed of the Blackman reaction.

Using 133 light flashes per second, Warburg obtained an improvement of 100 per cent over the continuous light yield. We were able to improve the continuous light yield 300 per cent to 400 per cent by using only 50 flashes per second and making the light flashes much shorter than the dark periods. This opened the possibility of determining the length of the dark period necessary for the complete removal of the intermediate product formed in a light flash of given intensity and duration. Lengthening the dark period should improve the yield until there is time enough for all the intermediate product formed in each light flash to be removed before the next light flash.

In this paper we describe experiments which show that the necessary dark time is about 0.03 to 0.4 of a second, depending on the temperature. Further experiments are described to show certain characteristics of the reactions taking place both in the light and in the dark.

I

Description of Electrical Circuits

Three types of lighting circuits were used, which we shall refer to as A, B, and C.

Circuit A, shown in Fig. 1, involved the use of a neon tube *N* about 12 inches long and $\frac{1}{4}$ inch thick, similar to those used in advertising. The tube was lighted by a transformer *T* whose primary was connected with the 110 volt 50 cycle alternating current of the laboratory. The secondary gave 15,000 volts on open circuit and 25 milliamperes on a short circuit. The neon tube was lighted about 95 per cent of the time. There was a momentary dark period each time the voltage changed sign.

In Circuit B a mercury rectifier bulb *B* was placed across the secondary of the transformer, as shown in Fig. 2. The filament of the rectifier bulb was lighted from a toy transformer, not shown in the diagram. A variable resistance *R* was put in series with the rectifier bulb. For this resistance one or more 5 watt 110 volt switchboard lamps were used, giving resistances up to 15,000 ohms.

Alternate half cycles of current were taken by the neon tube and the rectifier bulb, so that the neon tube flashed 50 times a second. The duration of each flash of the neon tube could be controlled within certain limits by varying R . With a high resistance, the tube was lighted about 45 per cent of the time, and with a low resistance about 15 per cent of the time. The per cent time of light

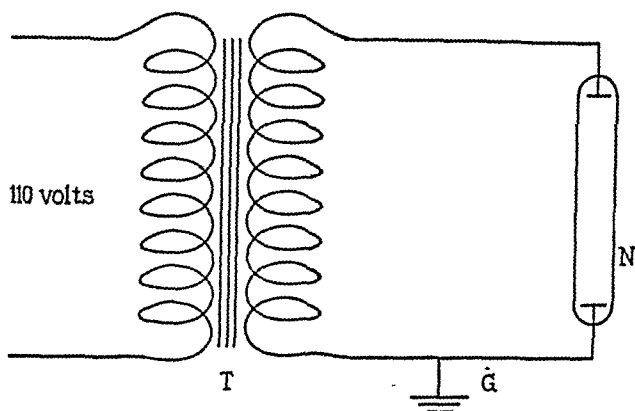


FIG. 1. Diagram of Circuit A

N = neon tube; T = transformer; G = ground

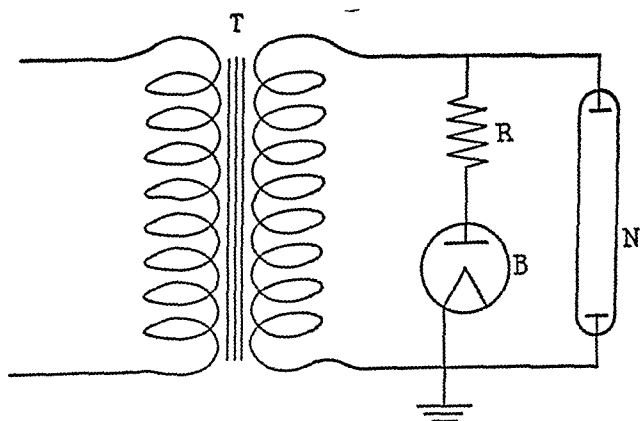


FIG. 2. Diagram of Circuit B. R = variable resistance; B = mercury rectifier bulb. Other letters are same as for Circuit A.

and dark was measured with the device shown in Fig. 3. A black disc about 4 inches in diameter, having a narrow strip of white paper along one radius, was spun on the shaft of a 4 pole synchronous motor, and the flickering light of the neon tube was thrown on the spinning disc by a mirror. Since the neon tube and the synchronous motor were operated from the same source of 50 cycle current, the light flashes kept pace exactly with the revolutions of the disc, and the

white paper radius was illuminated twice during each revolution. The spinning disc showed a stationary pattern of two dark sectors and two light sectors. By means of a protractor the angle subtended by one light sector was measured.

If this angle was x° , the per cent time of illumination was $\frac{x}{180} \times 100$.



FIG. 3. The rotating disc used to measure the duration of light and dark periods

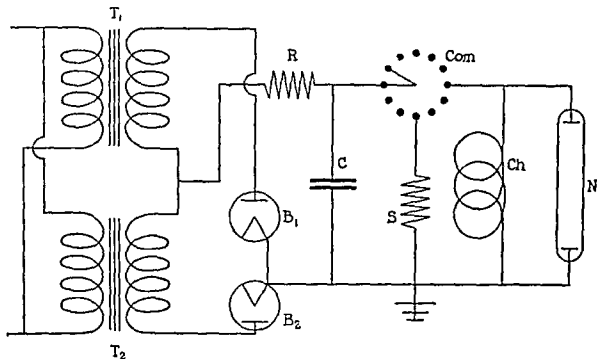


FIG. 4. Circuit C. *Com.* = commutator; *ch.* = choke coil; *S* = 600 watt electric stove; *C* = condenser; *R* = variable resistance. Other letters are same as for Circuit A.

For Circuit C a larger size of neon tube was used, about $\frac{5}{8}$ inch in diameter and again about 1 foot long. An extra large size of electrodes was used in these tubes. A diagram of the circuit is shown in Fig. 4. The half microfarad condenser *C* was charged from the full-wave rectifier system consisting of the two pole-top transformers *T*₁ and *T*₂ and two mercury rectifier bulbs *B*₁ and *B*₂ connected as

indicated. The filaments of the rectifier bulbs were lighted by a toy transformer. The transformers T_1 and T_2 were 110-2200 volt, and were rated one kilowatt each. The rate of charging of the condenser was controlled by the resistance R . When R was equal to 5000 ohms the condenser could be charged to well over the voltage necessary to strike the neon tube in half a cycle. At different times we used resistances of 2000 to 8000 ohms in the charging circuit.

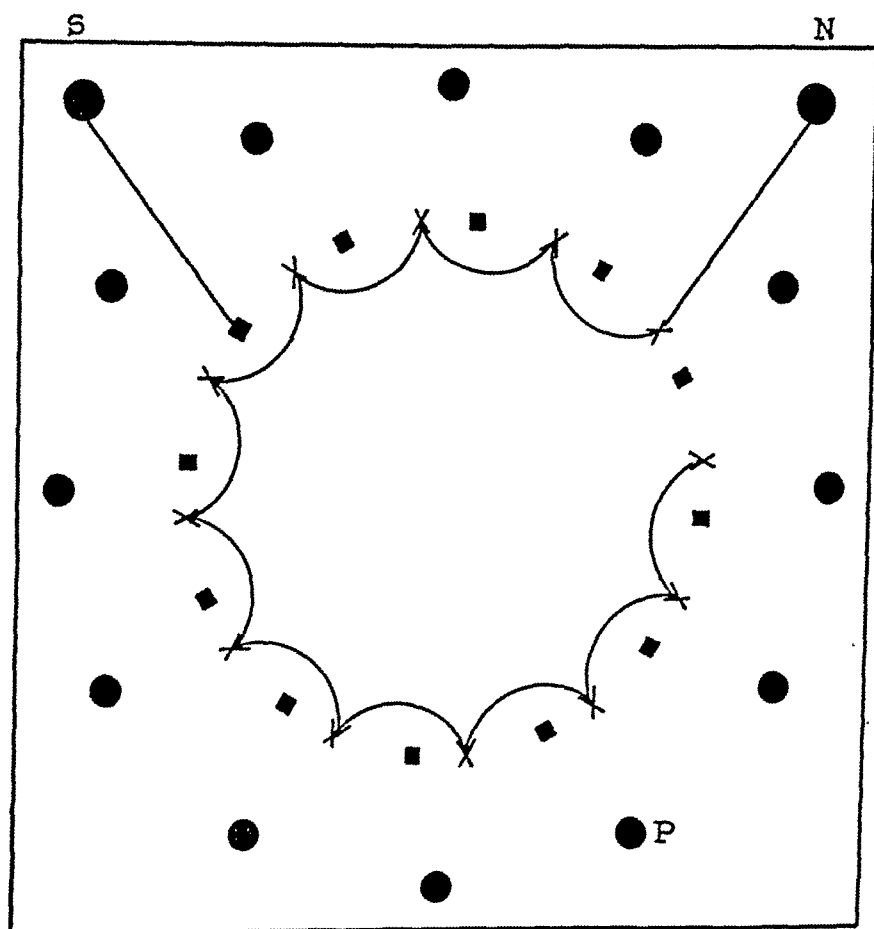


FIG. 5. Diagram of the commutator used in Circuit C. P = tungsten points; N is the binding post connected with the neon tube; S is the binding post connected with the electric stove.

Com. is the commutator which distributed the condenser discharges either through the neon tube N , or through the 600 watt electric stove S . A diagram of the commutator is shown in Fig. 5. It is a 9 inch square slab of bakelite on which twelve tungsten automobile ignition points P are mounted in a circle of about 4 inches radius. These tungsten points project about $\frac{1}{8}$ inch beyond the surface of the bakelite. By a system of switches not shown in the drawing each

point can be connected with either the neon tube or the electric stove. The crosses mark contacts connected by the wiring system shown, which leads out to the neon tube binding post. The squares are connected by a similar wiring system on the reverse side of the bakelite, leading out to the stove binding post. The rotor arm of the commutator also carries a tungsten point. This moves past each point on the commutator without touching it. The potential of 2000 volts is sufficient to break down the small air gap of about half a millimeter. The rotor arm is mounted on a reduction gear operated by the same synchronous motor used with Circuit B. In the course of the work we ran the rotor arm at speeds from 30 R.P.M. to 240 R.P.M. At 240 R.P.M. when every tungsten point on the commutator is connected with its cross contact the neon tube will flash 48 times a second. If every alternate tungsten point is connected with the square contact instead of the cross contact, the tube will light twenty-four times a second and twenty-four times the condenser will be discharged through the stove. Thus with a speed of the rotor arm of 240 R.P.M. the dark time between light flashes can be varied from 0.0208 second to 0.25 second, depending on whether twelve, six, four, three, two, or one contacts are connected with the neon tube. The purpose of connecting the points not used to light the tube with the electric stove is to allow the condenser the same charging time before each flash of light. Otherwise the longer dark periods would be followed by brighter flashes of light, since the condenser is not used at full charge.

When stating the dark time between flashes, we neglect the duration of the light flashes, which may be as short as 10^{-5} sec. We have made measurements which show that the flash lasts not longer than 2×10^{-5} sec. This was done by mounting a piece of white thread on a black wheel and rotating the wheel at 1500 R.P.M. while the flashing light was thrown on it. Each flash made the thread momentarily visible as a clear sharp image, not blurred perceptibly by motion. Even the twist of the thread could be seen. Assuming that the thread would just begin to look blurred if it moved half its diameter during the light flash, we can set an outer limit for the duration of the flash. The thread was about 0.2 mm. in diameter, and had a lineal velocity of 860 cm. per second. It would travel half its own diameter in about 2×10^{-5} sec. Since no blurring was visible, the duration of the flash was surely less than this. A calculation from the constants of the electrical circuit put the duration of the flash at about 1×10^{-5} sec. The time the light is on is therefore negligible compared to even the shortest dark period of 2.08×10^{-2} sec.

The time required for the tungsten point on the rotor arm to pass one point on the commutator is sufficient for the condenser to discharge once, and become charged again up to the striking voltage of the neon tube. This means that the first bright flash is followed closely by one or more dimmer flashes. These dim flashes stop as soon as the rotor arm moves clear of the contact in question. It was found that these secondary flashes could be prevented by the choke coil *Ch.*, a coil 5 cm. in diameter, 11 cm. long, and bearing 65 turns of No. 16 magnet wire. The steep wave-front from the well charged condenser takes the path through

the neon tube. The choke coil completely prevents the tube from lighting on any small voltage that may be set up in the system.

The discharge from a half microfarad condenser at 2000 volts subjects an ordinary neon tube to very severe strain. The tube does not last indefinitely. In time its inner walls become darkened from the copper deposited on them by the heavy current. The neon supply becomes so exhausted that the tube finally will not strike any longer. During these changes in the tube, the intensity of the flash may change considerably. This necessitates control of conditions at the close of each experiment, to make sure the tube will still produce the same photosynthesis as it did at the outset of the experiment.

II

The Technique of Measuring Photosynthesis

Photosynthesis was measured manometrically in rectangular glass vessels of about 10 cc. capacity, attached to Barcroft-Warburg manometers. Since the details of this method of measurement have already been adequately described in many different papers (Warburg, 1926) only those modifications which were necessitated by the use of the neon tubes are described here.

In order to obtain intense illumination of the cell suspensions, the neon tube was placed in the water of the thermostat a few millimeters below the bottoms of the vessels. The electrodes projected upwards at right angles to the main part of the tube toward the surface of the water. Fig. 6 shows the arrangement. *R, R* are pieces of rubber tubing slipped over the electrodes to prevent them from becoming wetted. *L* are the lead wires. *W* is the water level in the thermostat. *S* is the cell suspension in a rectangular glass vessel. Three such vessels could be placed over the tube at once, two with cell suspensions and one as a barometer.

When the light source is used so close to the vessels, great care must be taken that the two vessels containing the cells are the same distance from the tube, or else they will not be equally illuminated. This is very important in the flashing light, so either a given curve was made entirely with one vessel set always in the same position, or, if it was absolutely necessary to use two vessels at the same time, two were selected which would stand within a millimeter of the same distance from the tube, and which had bottom areas as nearly equal as possible. To insure that the vessels should receive light through their bottoms only, little copper jackets were made which covered the sides and tops. Without these copper jackets we found it impossible to obtain comparable sets of results.

For most of these experiments the unicellular green alga, *Chlorella pyrenoidosa* was used. The cells were produced in pure cultures as described in a paper by Emerson (1929, p. 611). The strain of *Chlorella* used for this work has now been in cultivation in our laboratory for 5 years and has shown no changes in metabolic activity.

Cells from well grown cultures were centrifuged out of the culture medium

and transferred after washing to a mixture of $M/10$ potassium carbonate and potassium bicarbonate solutions. The mixture used was 15 parts carbonate to 85 parts bicarbonate, except in experiments where low concentrations of carbon dioxide were required. Warburg (1919, p. 238) gives the properties of mixtures of $M/10$ sodium carbonate and bicarbonate. Although exact data are not available for the potassium carbonates, we have used them instead of the sodium salts because the cells seem able to withstand exposure to the potassium carbonate mixtures for a longer time without suffering a decrease in rate of photosynthesis. Although it is unlikely that the concentrations of carbon dioxide are the same as for the sodium mixtures, we have every reason to believe that they differ only

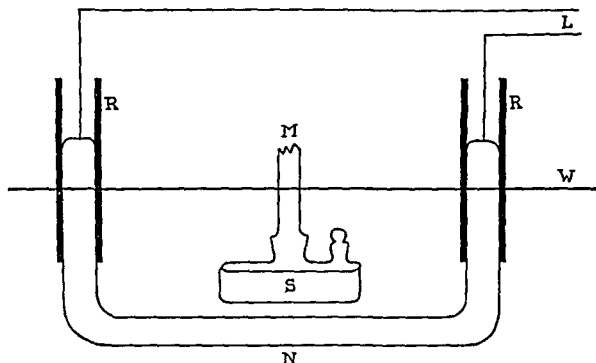


FIG. 6. Showing the positions of neon tube and vessel as used in the thermostat. *L* = lead-wires, one going to the commutator, the other to the condenser; *R* = rubber sheaths protecting electrodes; *W* = water level in thermostat; *S* = cell suspension; *N* = neon tube; *M* goes to the manometer tube.

by small amounts. Mixing the potassium solutions in the same proportions in which Warburg mixed his sodium solutions, we obtained curves closely similar to Warburg's which were made in the sodium mixtures.

Some experiments were made with the cells suspended in culture medium saturated with 5 per cent carbon dioxide in air, in order to make sure that the effects found were not connected with special properties of the carbonate mixture. In these cases it was necessary to use two vessels containing different volumes of cell suspension, as described by Warburg (1926, p. 108).

In order to ascertain whether the phenomena studied in flashing light were the same in other kinds of green cells, a few experiments were made with *Chlorella*

vulgaris, and with *Zostera marina*. The *C. vulgaris* was cultured in the same way as *C. pyrenoidosa*, and the *Zostera* was collected fresh from rock pools at low tide and used within 2 hours.

III

EXPERIMENTAL

As explained in the introduction, Warburg obtained a 100 per cent increase in photosynthesis from a unit amount of light by allowing the light to fall on the cells intermittently instead of continuously. He obtained his highest yields by using high frequencies of alternation of light and dark periods, while keeping the light and dark periods equal in length at any given frequency. To ascertain whether it was possible to still further increase the yield without further increasing the frequency, we used longer dark periods and shorter light periods. A neon tube connected as shown in Circuit B gave 50 flashes a second, and the flashes could be made to take up 45 per cent, 34 per cent, 25 per cent, or 17 per cent of the time. Readings were also taken with the tube burning only 7 per cent of the time, but the individual flashes were irregular in duration, so these readings are not included. As a standard of comparison for the photosynthesis in flashing light, we used the value of photosynthesis obtained with the neon tube connected as in Circuit A. As already stated, this does not give quite continuous light. The tube glows about 95 per cent of the time, and gives 100 flashes per second. However, photosynthesis was the same in this light as the maximum obtainable with ordinary incandescent bulbs (100 watt bulbs about 10 cm. from the cells), so we may regard the 95 per cent illumination as practically continuous as far as photosynthesis is concerned.

The results of an experiment carried out at 25°C. and at a relatively high concentration of carbon dioxide are shown graphically in Fig. 7, and numerically in Table I. The abscissae are *per cent time of illumination*, calculated from the angle measured on the rotating disc shown in Fig. 3. The ordinates are *photosynthesis per unit amount of light*. We found that for the shorter light periods the intensity was decreased, and since in flashing light photosynthesis becomes a linear function of intensity, we were obliged to make some correction for intensity differences for the different times of light and dark. We accomplished

this by comparing the readings at full intensity with others taken with a 5 per cent filter. At low intensities of light there is no improvement in photosynthesis with flashing light, and the rate is the integral of intensity \times time. The readings with no filter were divided by the corresponding readings with a 5 per cent filter to get photosynthesis per unit amount of light.

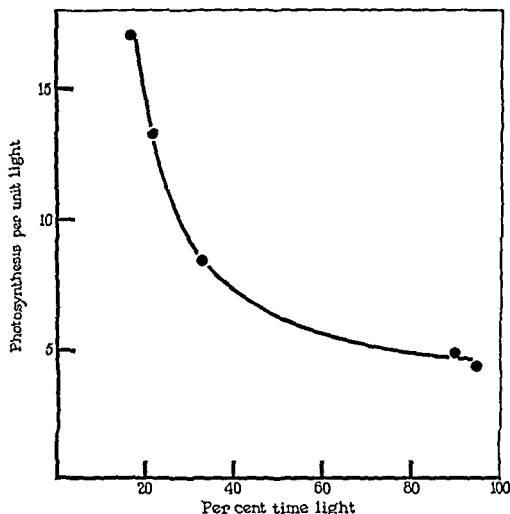


FIG. 7. Curve showing the relationship between per cent time of illumination and yield of photosynthesis per unit amount of light.

For 95 per cent light we have 4.4 units of photosynthesis for a given amount of light, and when the light is on only 17 per cent of the time, the same amount of light gives 17.7 units of photosynthesis. This is an improvement in yield of about 400 per cent. Extrapolation of the curve shows that still further improvement in yield could be obtained with longer dark periods and shorter light flashes.

From this experiment it is clear that the improvement in yield

is not only a function of the frequency of flashing. It is also dependent on the relative duration of light and dark periods. No information may be obtained from this experiment on the question of whether it is the increase in dark period or the shortening of the light flash which improves the yield. It may be either or both. We suppose that certain reactions in the photosynthetic process take place in the light only, and others can proceed in the light or for a short time in the dark. At 50 cycles per second one cycle is 0.02 second. 17 per cent of this is equal to 0.0034 second, the duration of one light flash when the light is on 17 per cent of the time. The corresponding dark period is 0.0166 second. Our light period of 0.0034 second is almost

TABLE I

Photosynthesis per unit amount of light with 50 flashes per second and different lengths of light and dark periods. Measurements made at 23.75°C. in carbonate mixture No. 9.

Time light in each cycle	Oxygen per hr. of light per c.mm. cells, 5 per cent filter (A)	Oxygen per hr. of light per c.mm. cells, no filter (B)	Photosynthesis per unit amount of light, no filter $\left(\frac{B}{A}\right)$
<i>per cent</i>	<i>c.mm.</i>	<i>c.mm.</i>	
95	4.04	17.8	4.4
90	3.65	18.1	4.9
33	1.93	16.4	8.5
22	0.84	11.2	13.3
17	0.44	7.9	17.7

the same as Warburg's shortest period of 0.0038. Our improvement in yield is 400 per cent as compared to his of 100 per cent. Our dark period is about 4.5 times as long as his. We can say from this that surely the longer dark period plays a large part. Our next experiment was designed to show how long a dark period was necessary to get the best possible yield out of a light flash of given intensity and duration.

For this it was necessary to use light flashes always of the same intensity and duration, but separated by a variable dark period. Our short light flashes were obtained from Circuit C, and our dark periods were timed by the commutator shown in Fig. 5.

The amount of photosynthesis per flash of light was calculated from the number of flashes per second and from the photosynthesis

in 5 minute periods, properly corrected for respiration. The photosynthesis per flash was plotted against the dark time between flashes. Fig. 8 shows three series of results plotted in this way. All were made with cells from the same culture. The points plotted as open circles were made first, at a temperature of 25°C. The ordinates

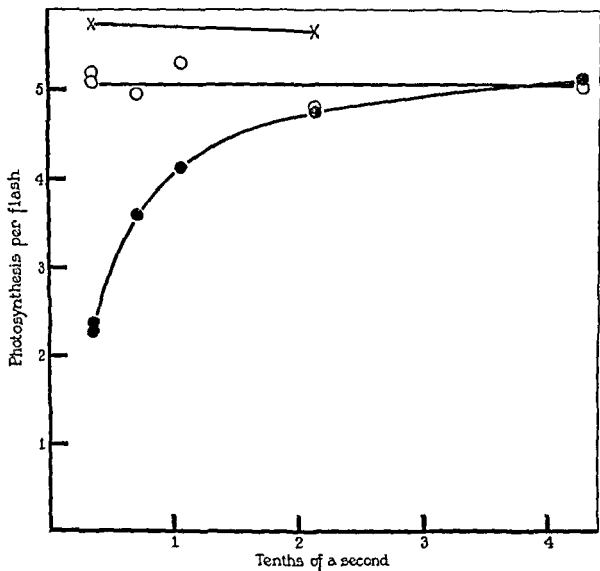


FIG. 8. The effect of dark time on yield of photosynthesis per flash of light. Open circles are points made at 25°C., solid circles at 1.1°C. The crosses are a check made at 25°C.

are photosynthesis per flash, and the abscissae the dark periods in tenths of a second. The shortest dark period used, about 0.04 second, is adequate for the complete removal of the material remaining at the end of each light flash. The longest dark period shown on this figure is over 0.4 second, and the yield per flash after this dark period is no

greater than the yield per flash after 0.04 second. We have tried lengthening the dark period to over 2 seconds, without finding any increase in photosynthesis per flash.

The points indicated by closed circles, plotted on the same scale as the open circles, were made at 1.1°C. Here the yield per flash is much lower with the short dark periods, but rises with increasing dark time until, after 0.4 second it is equal to the yield at 25°.

The crosses are measurements made at 25°C., after the completion of the curve at 1.1°C., to make sure that the original results obtained at 25°C. could be duplicated.

In considering these curves, and others like them to be described in this paper, it must be remembered that the light is "on" for a relatively small proportion of the time which elapses between readings. Consequently the dark reading used to correct for respiration plays a more important part than in continuous light. The longer the dark periods the smaller is the reading obtained for photosynthesis, and the more does the accuracy of the photosynthesis determination depend on the accuracy of the respiration reading. Respiration is never very accurately determined in the thin cell suspensions used for studying photosynthesis, and the respiration during light periods, either continuous or intermittent, may differ slightly from the respiration in an unbroken dark period, the only time when we can measure respiration. A small error in respiration will displace the points at the left hand ends of the curves only slightly, but will displace the right hand points much more, because there are only one-twelfth as many light flashes per unit time for the right hand readings as for the left hand readings. It is most favorable to work at low temperatures, where respiration is almost zero.

The numerical values of the points plotted in Fig. 8 are given in Table II. Protocol 1 at the end of the paper gives all experimental data. This experiment shows that the dark process is temperature-sensitive, and that the light process is not. If the light process were also temperature-sensitive, Curve B would not attain the same level as Curve A. Since that part of the photosynthetic process which is temperature-sensitive is usually called the Blackman reaction, we feel justified in saying that it is the Blackman reaction which goes on in our dark period, without implying whether or not other reactions are involved as well.

These curves would be the same in shape whether the Blackman reaction preceded or followed the photochemical reaction. In the first case the long dark period would improve the yield because *preparation* for the light reaction had time for completion; in the second case because changes *following* the light reaction had time for completion. Our experiments offer no decision on this point, but since Warburg (1920, p. 188, Section VI) has presented evidence that the Blackman reaction *follows* rather than *precedes* the photochemical reaction, we shall speak of the dark process as following the light process.

TABLE II

Photosynthesis per flash of light after different lengths of dark period. The light flashes were kept constant and only the dark periods varied. Complete data in Protocol I.

Dark time	Oxygen per flash per c.mm. cells $\times 10^3$		
	At 24.85°C.	At 1.1°C.	At 24.87°C.
<i>sec.</i>	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>
0.035	5.20	2.28	5.75
0.425	5.04	5.12	
0.212	4.80	4.75	5.64
0.106	5.30	4.14	
0.071	4.97	3.60	
0.035	5.10	2.39	

The light reaction, we suppose, produces an intermediate product. This product might either be converted to other intermediate or final products of photosynthesis through the operation of the Blackman reaction, or it might spontaneously revert to its original state or component parts. Since the curve plotted with solid circles in Fig. 8, made at 1.1°C., attains after 0.4 second the same level as that shown by the open circles after 0.03 second, made at 25°C., it is clear that the slower rate of the Blackman reaction at 1.1°C. has resulted in no loss of intermediate product. There has merely been a delay in the utilization of the product of the photochemical reaction. Therefore if any spontaneous decomposition of the intermediate product formed by the photochemical reaction takes place, the loss must be the same after 0.03 second at 25°C. as after 0.4 second at 1.1°C. In other words, the temperature coefficient of the spontaneous decom-

position must be the same as the temperature coefficient of the Blackman reaction. This is an unlikely possibility, and we consider it more probable that no spontaneous decomposition takes place. This is supported by experiments in which the Blackman reaction was inhibited by prussic acid. It is most improbable that the prussic acid would have the same specific effect on both the Blackman reaction and any spontaneous decomposition or deactivation.

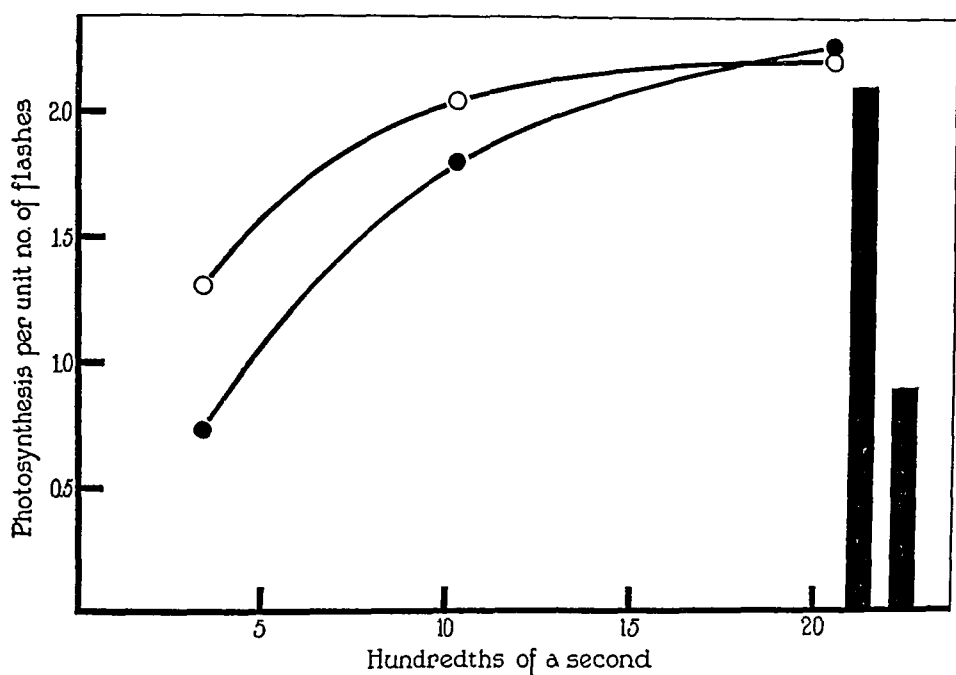


FIG. 9. The effect of prussic acid on the course of the dark reaction. Open circles are points made without prussic acid, solid circles points made with 1.14×10^{-5} mols prussic acid per liter. The black columns show the effect of the same concentration of prussic acid on photosynthesis in continuous light. The high column is the rate without prussic acid, and the short column is the rate when inhibited by prussic acid.

We used a free prussic acid concentration of 1.14×10^{-4} mols per liter in Warburg's carbonate mixture No. 9. (For the calculation of free prussic acid in carbonate mixture containing potassium cyanide see: Emerson, 1929, p. 635.) This concentration of 1.14×10^{-4} mols per liter is sufficient to inhibit photosynthesis in continuous light 60 per cent. In flashing light the longer the dark periods the

less the inhibition, and if the dark periods are long enough the cells in cyanide will give the same value as the cells without cyanide. This is shown graphically in Fig. 9. The cyanide curve plotted with solid circles slopes upward more steeply than the normal curve, which is plotted with open circles. After 0.2 second the cyanide curve has reached the level of the normal, and there is no inhibition. Changing to continuous light there appears immediately a 60 per cent inhibition of photosynthesis, shown graphically by the black columns. The numerical values for Fig. 9 are given in Table III. Photosynthesis has not been reduced to the value per single flash of light, but only

TABLE III

The effect of cyanide on the course of the dark reaction, and on photosynthesis in continuous light. Measurements made in carbonate mixture No. 9, at a temperature of 13°C., using 9.6 c. mm. cells suspended in 7 cc. of fluid.

Dark time	Oxygen per unit No. of flashes		Inhibition by HCN
	Without cyanide	Free HCN 1.14×10^{-4} mols per liter	
<i>sec.</i>	<i>c. mm.</i>	<i>c. mm.</i>	<i>per cent</i>
0.035	1.31	0.74	50
0.106	2.04	1.80	12
0.212	2.20	2.26	0
5 min. continuous light.....	8.43	3.60	57.

to the value per unit number of flashes. For convenience the unit used was the number of light flashes in 5 minutes when the neon tube was flashed once (or in some cases twice) in each revolution of the commutator. It was not thought necessary to reduce the figures to photosynthesis *per* single flash except in the case of Table II and Fig. 8.

Fig. 9 shows that the Blackman reaction requires a longer time to complete its work in the presence of cyanide, but the total amount of photosynthesis done is no less in the cyanide if the dark periods are long enough. We can say then that whatever is produced in the light reaction is stable for at least 0.4 second, and is removed only through the operation of the Blackman reaction.

It might be that chlorophyll alone is involved in the light reaction, and the absorbed energy transferred later to a carbon dioxide compound, or it might be that both chlorophyll and carbon dioxide are required for the light reaction. In the first case we would expect that lower carbon dioxide concentrations would not decrease the yield per flash but would necessitate longer dark periods for full utilization of the light. In the second case we would expect the yield per flash to be cut down by low carbon dioxide concentrations, but the course of the dark reaction should remain unchanged.

TABLE IV

A comparison of the effect of carbon dioxide concentration on photosynthesis in continuous and intermittent light. Each measurement made with 8.2 c. mm. cells, at a temperature of 24.27°C. Carbon dioxide concentrations are for sodium mixtures.

Carbon dioxide concentration in mols per liter $\times 10^{-4}$ (from Warburg)	Oxygen produced in 5 min. continuous light	Oxygen produced in 5 min. flashing light, 24.05 flashes per sec.
	<i>c. mm.</i>	<i>c. mm.</i>
2.6	4.46	2.80
9.8	9.84	3.62
23.0	15.55	4.36
91.0	20.35	5.44
91.0	20.97	5.92
23.0	16.05	4.85
9.8	9.20	3.80
2.6	3.00	2.46

Before making this experiment it was necessary to test whether the process of photosynthesis is the same function of carbon dioxide concentration in flashing light and in continuous light, in order to select appropriate concentrations. This was done at a high temperature and with dark periods of about 0.1 second to allow for completion of the dark reaction between flashes. Table IV shows the rates of photosynthesis in four different carbonate mixtures in both flashing and continuous light. The values are plotted against carbon dioxide concentration in Fig. 10. The points shown by open circles were made in continuous light, and those shown by solid circles in intermittent. The upper curve is similar in shape to the lower curve.

Carbon dioxide saturation is approached in the same way in continuous and in flashing light. In carbonate mixture No. 9 the rate is changing only very slowly with carbon dioxide concentration, and in mixture No. 5 the rate is cut down about half, and varies proportionately to concentration. Complete data for this experiment are given in Protocol II.

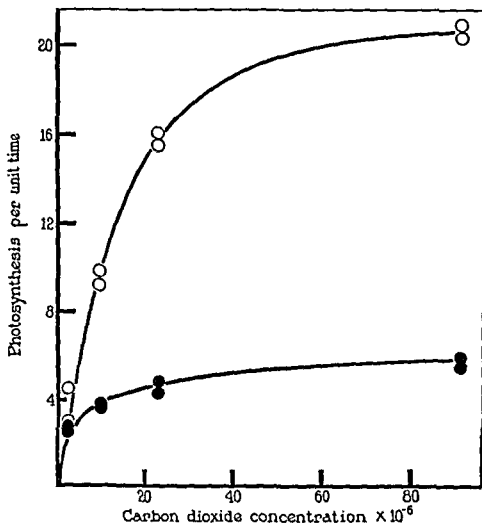


FIG. 10. The effect of carbon dioxide concentration on photosynthesis in continuous light (open circles) and in flashing light (solid circles), twenty-four flashes per second.

The course of the dark reaction was studied in these two mixtures at 6°C. Table V gives the relative values of photosynthesis per flash for the various dark periods, and the results are plotted in Fig. 11. The upper curve is for the higher carbon dioxide concentration, 71×10^{-6} mols per liter, and the lower curve for the lower concentration, 4.1×10^{-6} mols per liter. Both curves bend parallel to the abscissa

after a dark time of 0.08 second. There is no indication that the lower curve will rise to the level of the upper one. The lower concentration of carbon dioxide prevents the light flashes from producing as much photosynthesis as at high concentrations, regardless of dark time. The dark reaction apparently runs its course in about the same length of time, regardless of the amount of intermediate product produced by the light flash.

This experiment shows that the carbon dioxide enters the process of photosynthesis either before or coincident with the photochemical reaction. It seems unlikely that the carbon dioxide molecules could

TABLE V

The course of the dark reaction at two concentrations of carbon dioxide. For each measurement 9.0 c. mm. cells were used. Temp. = 5.9°C.

Dark time	Oxygen produced per unit No. of flashes	
	CO ₂ conc. 4.1×10^{-6} mols per liter	CO ₂ conc. 71×10^{-6} mols per liter
sec.	c.mm.	c.mm.
0.02	0.91	2.34
0.04	2.11	3.89
0.08	2.89	4.92
0.12	2.78	5.13
0.02	1.14	2.36

move into position and react during the extremely short light flash of about a hundred-thousandth of a second. We think it more reasonable to suppose that the carbon dioxide reacts first, possibly combining with chlorophyll in the dark before the light flash. The concentration of chlorophyll-CO₂ formed would depend on the concentrations of chlorophyll and of carbon dioxide. The light flash would activate the chlorophyll-CO₂ molecules and leave them ready to undergo the Blackman reaction. The possibility that carbon dioxide combines with chlorophyll in photosynthesis has already been suggested by Willstätter (1918, p. 172). If such a compound is formed we would expect that low chlorophyll concentrations would affect the course of the dark reaction like low concentrations of carbon dioxide.

The chlorophyll content of *Chlorella pyrenoidosa* cells cannot be

varied conveniently with the method used by Emerson (1929) for *Chlorella vulgaris*, but it was found possible to change the chlorophyll concentration per unit volume of *C. pyrenoidosa* cells by another method. Details and quantitative data will probably be given in a later paper. When cultures are grown over red (neon) and blue (mercury) luminous tubes, the cells produced in red light contain roughly one-fourth the chlorophyll of those produced in the blue light.

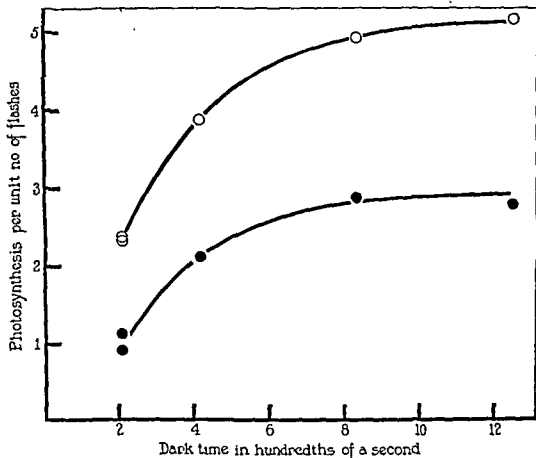


FIG. 11. The course of the dark reaction at two different concentrations of carbon dioxide. Open circles, CO₂ concentration 71×10^{-6} mols per liter. Solid circles, CO₂ concentration 4.1×10^{-6} mols per liter.

A unit volume of cells grown in blue light will photosynthesize almost four times as fast as the same volume of cells grown over red light, when both kinds are used over continuous incandescent light.

Table VI gives data for the course of the dark reaction with these two types of cells. Photosynthesis per unit number of flashes is plotted against dark time in Fig. 12. Owing to the much smaller values for the cells grown in red light these points, plotted as solid

in continuous light about 50 per cent. The phenylurethane could be weighed out and dissolved conveniently, but the thymol was so insoluble that we were obliged to saturate a solution at a given temperature by adding excess thymol, warming, and filtering after the solution had cooled to the temperature desired. The filtered solution was then diluted until we found a concentration giving 50 per cent inhibition.

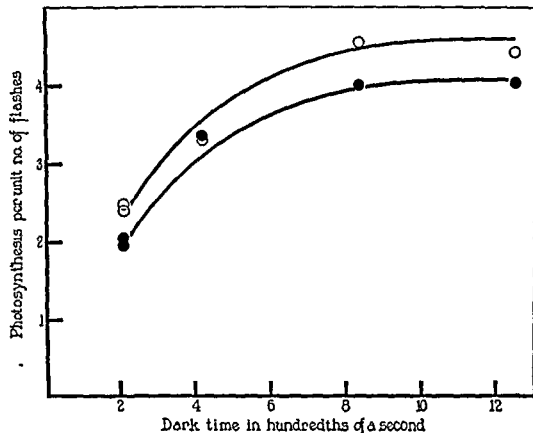


FIG. 12. The course of the dark reaction at a low concentration of chlorophyll (open circles) and at a high concentration of chlorophyll (solid circles). The observations for the open circles have been multiplied by a constant to bring the curves close together and make them easily comparable.

A narcotic concentration which inhibits photosynthesis 50 per cent in continuous light will give about 50 per cent inhibition in flashing light if the flashes are far enough apart to allow for completion of the Blackman reaction. Neither thymol nor phenylurethane had a marked effect on the Blackman reaction. In order to make the effect clearer, the cells inhibited by narcotic were compared with others whose photosynthesis was cut down by a 50 per cent filter, so that in each

case the Blackman reaction would have about the same amount of intermediate product on which to work. It should be mentioned that the rate of photosynthesis in flashing light is almost a linear function of intensity. The work on intensity is not yet completed, and will be published later.

Figs. 13 and 14 show the course of the dark reaction in narcotic and with a 50 per cent transmission filter. The narcotic curves rise

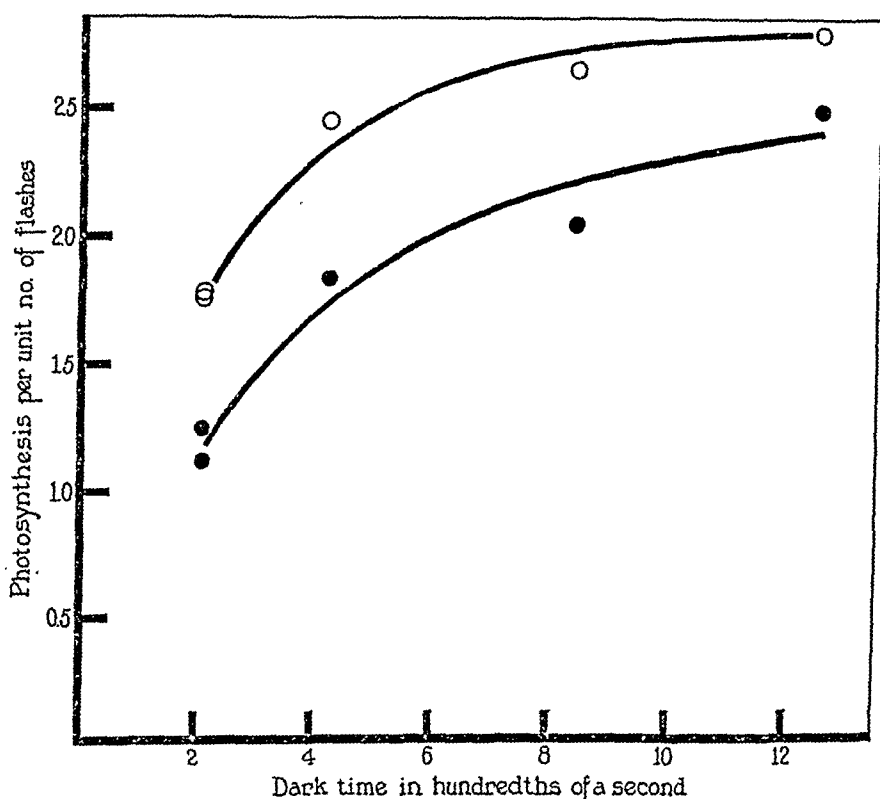


FIG. 13. The effect of phenylurethane on the course of the dark reaction. Open circles are points made without urethane, solid circles points made with urethane 0.0034 per cent.

slightly more gradually and for a longer time than the curves for the 50 per cent filter, indicating that the Blackman reaction may be slightly inhibited by narcotics. However, the chief effect of narcotics is an inhibition of the formation of intermediate product, possibly by preventing some of the chlorophyll from combining with carbon dioxide, or possibly by a direct inhibition of the photochemical

reaction. This would also explain why photosynthesis at both high and low intensities is inhibited by narcotics, even though the Blackman reaction is not much affected.

To make sure that the phenomena described in this paper were not special characteristics of *Chlorella pyrenoidosa*, some experiments were made with *Chlorella vulgaris* and with *Zostera marina*. Increase of yield with increasing dark time could be demonstrated in both organ-

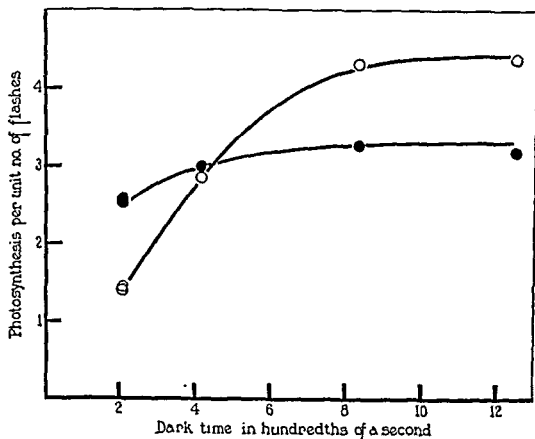


FIG. 14. The effect of thymol on the course of the dark reaction. Solid circles are points made without thymol, open circles points made with 1/70 saturated thymol.

isms, using the same intensity of light, temperature, and dark periods as for *Chlorella pyrenoidosa*. The course of the dark reaction for *C. vulgaris* (solid circles) is plotted with a curve for *C. pyrenoidosa* (open circles) in Fig. 15. The curves were made at the same time under identical conditions. The cells of *C. vulgaris* are much larger than *C. pyrenoidosa*, the chloroplasts are different, the chlorophyll content smaller, the rate of growth slower, and the relative values of

TABLE VII

Effect of narcotics on the course of the dark reaction. Temperature 7°C., cells suspended in carbonate mixture No. 9.

Dark time	Oxygen produced per unit number of flashes		
	With 50 per cent filter, no narcotic	With 100 per cent filter, and narcotic	
<i>sec.</i>	<i>c.mm.</i>	<i>c.mm.</i>	
0.02	1.77	1.25	0.0034 per cent phenyl- urethane, 13.5 c.mm. cells
0.04	2.45	1.84	
0.08	2.64	2.05	
0.12	2.75	2.48	
0.02	1.78	1.12	
0.02	2.54	1.42	1/70 saturated thymol, saturated at 21°C., 16.5 c.mm. cells
0.12	3.18	4.37	
0.08	3.26	4.30	
0.04	3.00	2.81	
0.02	2.58	1.45	

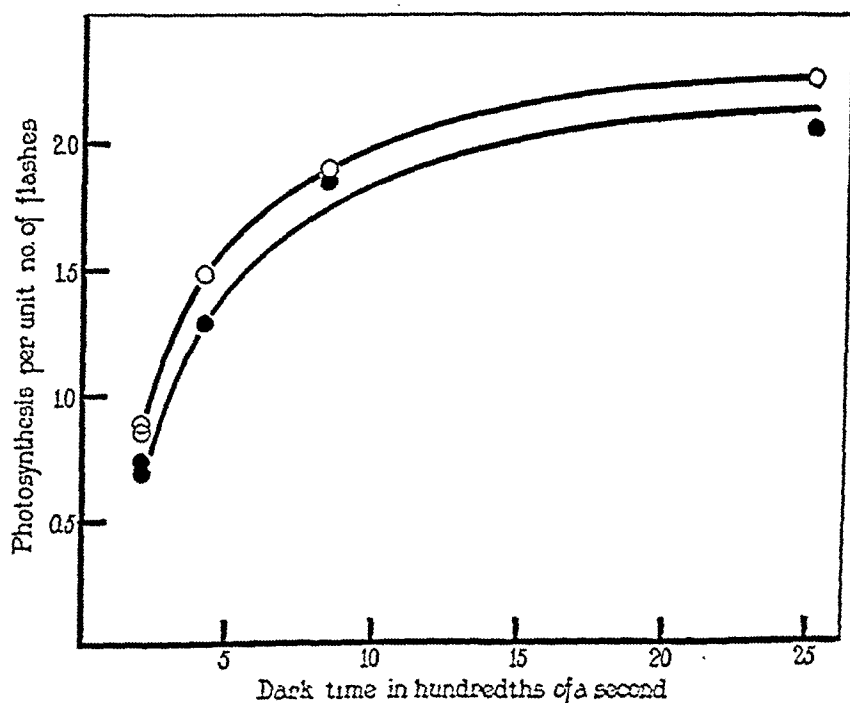


FIG. 15. A comparison of the course of the dark reaction in *Chlorella pyrenoidosa* (open circles) and *Chlorella vulgaris* (solid circles).

photosynthesis and respiration are different. The similarity of the curves is striking, and we believe this relationship between light and dark reactions is probably a general property of chlorophyll photosynthesis.

We have also measured photosynthesis in flashing light with cells suspended in phosphate solution saturated with 5 per cent carbon dioxide in air, to make sure that the carbonate mixture was not responsible for our curves. The photosynthetic quotient was found to be unity, and the effect of lengthening the dark time was the same as in carbonate mixture No. 9.

TABLE VIII

A comparison of the dark reactions of *Chlorella pyrenoidosa* and *Chlorella vulgaris*. Cells suspended in carbonate mixture No. 9 at a temperature of 6.5°C.

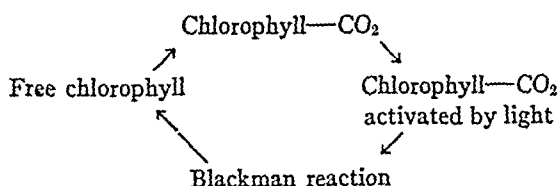
Dark time	Oxygen produced per unit No. of flashes	
	8.75 c.mm. <i>C. pyrenoidosa</i>	20.5 c.mm. <i>C. vulgaris</i>
sec.	c.mm.	c.mm.
0.02	0.86	0.69
0.04	1.48	1.29
1.08	1.90	1.85
0.12	1.78	1.83
0.25	2.25	2.05
0.02	0.88	0.74

VI

RECAPITULATION

The experiments described in this paper show that photosynthesis involves a light reaction not affected by temperature, and capable of proceeding at great speed, and a dark reaction dependent on temperature, which requires a relatively long time to run its course. The light reaction can take place in about a hundred-thousandth of a second. The dark reaction requires less than 0.04 second for completion at 25°C., and about 0.4 second at 1.1°C. The light reaction is dependent on carbon dioxide concentration and is inhibited by narcotics. The dark reaction is not noticeably inhibited by narcotics, is independent of carbon dioxide concentration, and is strongly inhibited by cyanide.

Throughout the paper the results have been treated on the supposition that the Blackman reaction follows the photochemical reaction. This is true in the sense that a given carbon dioxide molecule undergoes first the photochemical reaction and then the Blackman reaction. But it is likely that the Blackman reaction also sets free the chlorophyll, enabling the latter to react again with carbon dioxide. Emerson (1929) has presented evidence that the chlorophyll is involved in the Blackman reaction. To this extent, then, the question of whether the photochemical reaction precedes the Blackman reaction is only an academic one. From the point of view of the carbon dioxide the photochemical reaction comes first, but the process may equally well be regarded as a cycle in which free chlorophyll combines with carbon dioxide, becomes activated by light, undergoes the Blackman reaction, and is again set free to combine with more carbon dioxide:



Our thanks are due to various members of the electrical engineering and physics staff of the California Institute, and especially to Professor MacKeown and Professor Bowen, for much helpful advice on the electrical circuits. We are also indebted to Mr. Erickson of the Electrical Products Corporation for his advice on the type of tubes and electrodes best suited to our work, and to the Electrical Products Corporation for supplying the tubes.

Protocol I

Complete Data for Fig. 8 and Table II

All measurements made in same vessel. *C. pyrenoidosa* suspended in 7 cc. carbonate mixture No. 9.

Rotor arm making 141 R.P.M.

Resistance in charging circuit 7500 ohms.

Half-wave rectifier, 2200 volts, used to charge condenser.

No. of flashes per revolution of rotor arm	Dark time	Date...	Aug. 9		Aug. 10		Aug. 11	
		Temp..	24.85°C.		1.1°C		24.87°C.	
		K_2O_3 ...	0.41		0.464		0.41	
		C.mm. cells.	10.4		9.0		10.3	
		Change in pressure in 5 min.	Oxygen per flash per c.mm. cells $\times 10^3$		Change in pressure in 5 min.	Oxygen per flash per c.mm. cells $\times 10^3$		Change in pressure in 5 min.
	sec.	mm.	c.mm.		mm.	c.mm.	c.mm.	mm.
12	0.035	+9.33	5.20		+3.50	2.28	+10.27	5.75
1	0.425	-0.93	5.04		+0.43	5.12		
2	0.212	-0.10	4.80		+1.04	4.75	+0.03	5.64
4	0.106	+1.97	5.30		+2.00	4.14		
6	0.071	+3.50	4.97		+2.70	3.60		
12	0.035	+9.03	5.10		+3.67	2.39		
Dark respiration		-1.83			-0.27		-1.97	

Protocol II

Complete Data for Fig. 10 and Table IV

The effect of carbon dioxide concentration on photosynthesis in continuous and flashing light. Temperature 24.27°C. 8.2 c. mm. cells suspended in 7 cc. carbonate mixture used for each determination.

$K_2O_3 = 0.41$. Resistance in charging circuit 5000 ohms.

No of mixture	Composition of mixture		Concentration of CO ₂ in mols per liter*	Continuous light			24 flashes per sec.		
	$\frac{M}{10}$ K ₂ CO ₃	$\frac{M}{10}$ KHCO ₃		Change of pressure, 5 min.		O ₂ per hr. per c.mm. cells	Change of pressure, 5 min.		O ₂ per hr. per c.mm. cells
				Light	Dark (respiration)		Light	Dark (respiration)	
	cc.	cc.		mm.	mm.	c.mm.	mm.	mm.	c.mm.
4	70.	30.	2.6	+5.47	-1.97	4.46	+2.70	-1.97	2.80
6	50.	50.	9.8	+14.50	-1.93	9.84	+4.10	-1.93	3.62
7	35.	65.	23.0	+23.75	-2.17	15.55	+5.10	-2.17	4.36
9	15.	85.	91.0	+31.80	-1.57	20.35	+7.50	-1.57	5.44
9	15.	85.	91.0	+33.37	-1.57	20.97	+8.30	-1.57	5.92
7	35.	65.	23.0	+24.60	-2.17	16.05	+5.93	-2.17	4.85
6	50.	50.	9.8	+13.45	-1.93	9.20	+4.40	-1.93	3.80
4	70.	30.	2.6	+3.05	-1.97	3.00	+2.15	-1.97	2.46

* Figures from Warburg for sodium carbonate mixtures.

Protocol III

Complete Data for Fig. 11 and Table V

The course of the dark reaction at two concentrations of carbon dioxide.

9.0 c. mm. cells suspended in 7 cc. of carbonate mixture used for each measurement. $KO_2 = 0.453$. Temperature = $5.9^\circ C$.

Resistance in charging circuit 5000 ohms.

Rotor arm turning 240 R.P.M.

No. of flashes per revolution of rotor arm	Dark time	Carbonate mixture 5, 60 cc. $\frac{M}{10} K_2CO_3$, 40 cc. $\frac{M}{10} KHCO_3$, 4.1×10^{-3} mols CO_2 per liter		Carbonate mixture 9, 15 cc. $\frac{M}{10} K_2CO_3$, 85 cc. $\frac{M}{10} KHCO_3$, 71×10^{-3} mols CO_2 per liter	
		Change of pressure, 5 min.	O_2 per unit No. of flashes	Change of pressure, 5 min.	O_2 per unit No. of flashes
	sec.	mm.	c.mm.	mm.	c.mm.
12	0.02	+4.45	0.91	+13.25	2.34
6	0.04	+5.35	2.11	+10.85	3.89
3	0.08	+3.30	2.89	+6.55	4.92
2	0.12	+1.75	2.78	+4.30	5.13
12	0.02	+5.80	1.14	+13.30	2.36
Dark reading, respiration...		-1.03		-0.83	

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ANALYSIS OF THE GEOTROPIC ORIENTATION OF YOUNG RATS. V

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I

Findings recorded in several earlier papers¹ have indicated the suggestive possibilities of the method of testing the theory of the geotropic orientation of young rodents by forcing them to carry added loads. In young rats the sensorially equivalent bilateral excitation of tension-receptors excited during stepping determines the angle θ of oriented progression on a surface of inclination α .¹ The distortion of the curve connecting θ with α by the attachment of a small mass at a given position on the rat is entirely homologous in the case of two races of rats for which the respective θ - α curves are quite unlike. The attachment of the same mass at a different position produces a quite different deformation of the θ - α curve. The interpretation of these effects¹ has been given in terms of the manner in which the attached weight modifies the excitation of the fundamentally tripartite distribution of thresholds for excitation of the tension receptors concerned. The experiments have involved (a) placing a weight of 2.12 gm. on the back of young rats at hip level, and (b) placing the same mass at shoulder level. It has been pointed out that the extension of these experiments may permit one to locate anatomically the spatially distinct groups of receptor units clearly acted upon differentially by the differently placed additional masses. Qualitatively, the effect of an added load is similar to that in certain invertebrates (Crozier and Stier, 1928-29; Kropp and Crozier, 1928-29; Barnes, 1930; Hoagland and Crozier, 1931-32), and in the guinea pig (Upton, 1931-32). The point which now concerns us is the more detailed utilization of the effect as an aid to the localization of the receptors influential in determining θ as a function of slope of surface.

¹ Crozier and Pincus, 1926-27; 1929-30, b; 1931-32, b.

This involves careful scrutiny of the form of the θ - α curve. When an additional load of 2.12 gm. is carried at shoulder level by rats 13 to 14 days old, of our race A,¹ the orientation-angles at low values of α are *below* those obtained without the added weight, and at higher slopes *above* these angles. The form of the θ - α curve shows (Crozier and Pincus, 1931-32, b) that a small "additional" group of receptors is brought into play, at slopes of surface above $\alpha = 30^\circ$, not perceptibly implicated when no added weight is carried nor when the weight is posteriorly located. The fact that whether the added mass is located anteriorly or posteriorly determines whether the orientation-angle shall be increased or decreased, by comparison with the curve for unloaded individuals at the same slope of surface, is sufficient to show the necessity for further analysis of the curve of orientation-angles. We have pointed out that in all probability the *anteriorly* situated additional mass, by moving the centroid of the total weight to a more anterior spot higher from the substratum, must, at adequate slopes of surface, force higher orientations (θ) by endangering mechanical stability unless greater orientation is attained. This can be tested by dividing the added load in halves, one of which is affixed at hip level, the other in the "anterior position" at the shoulders. None of the effects described can be attributed *merely* to increase in the total weight involved. There is no correlation between orientation-angles and weight of individual, as we have already demonstrated. Other measurements made with litters of ages 13 to 24 days (the method of progression remaining comparable) show no changes of this character, nor any disturbance of the basic relation between θ and α , although the weight of the animal has increased considerably; the reason for this does not concern us at the moment. The way in which the whole procedure is checked by the analysis of variation of performance (*i.e.*, of θ) has been described.¹ This last is important because it is technically difficult to secure attachment of the added loads to different rats in exactly comparable places.

It has been of interest to endeavor to predict the outcome of placing 1.05 gm. both posteriorly and anteriorly on the back. The total load is then the same as when 2.1 gm. is carried in either position. But the torque effect due to shift of centroid should be largely eliminated. By comparison with the curve for unweighted individuals we

then expect that (1) the threshold slope for significant orientation should be lowered; (2) at the new, lowered threshold (if accurately enough located) the orientation-angle should be that characteristic of the unweighted animals at their threshold slope; (3) for equivalent θ 's the corrected S.D. of mean θ should be the same, for weighted and unweighted individuals; (4) the indices of variation should be the same for the two series; and (5) (save perhaps at the very highest workable slopes) the values of mean θ should be constantly above those of the curve for orientation without weights, but not so high at low α 's as with the posterior weight (2.1 gm.) alone. The reasons for these expectations are contained in the interpretation of the curve of distribution of thresholds for the tension-receptors concerned, and in the analysis of the thresholds and the variation of performance as governed by the numbers of these receptor units (Crozier and Pincus, 1931-32, *b*).

II

Three series of experiments were made, using the procedure earlier outlined (Crozier and Pincus, 1931-32, *a*). The data are set out in Table I and in Fig. 1. They show at once that reproducible series are obtainable, in spite of the difficulty of adjusting the additional loads. This difficulty was greatest with the first series. They also show that the threshold slope for significant orientation has been lowered from *ca.* $\alpha = 20^\circ$ to $\alpha = 15^\circ$ by the weights, and that mean θ at the new threshold is quantitatively equivalent to that without the presence of the weights. The curve lies, at low α 's, below that for rats with the same *total* load (2.1 gm.) located posteriorly. Comparison of these series with those previously given (Crozier and Pincus, 1931-32, *a*) for unweighted *A* rats shows that when N and n are equal, P.E. _{θ} is essentially the same for corresponding magnitudes of θ . The variability number ($[\Delta \text{P.E.}_\theta / \theta] / [\Delta \theta]$) for the series with two weights is given in Table II, and the proportionate modifiable variation. The graphs from which these and related quantities are computed are given in Figs. 2-8.

Table II shows that the variability number computed in the usual way (V.N.) is of the same magnitude as in *A* animals without load, but is notably higher in Series I; Series II and III, as already intimated,

were held to be more reliable; the total variation (area under the variability graph, Figs. 2, 6) is distinctly higher in the case of I.

TABLE I

Orientation-angles (θ) for young rats of line A, creeping geotropically on a surface of inclination α , with 1.05 gm. attached dorsally at shoulder level and 1.05 gm. at hip level. (For data on the orientation of *unweighted* rats of line A see: Crozier and Pincus, 1931-32, *a*, Table I). The subscripts indicate the number of the close-inbred generation. Series I, 3 ♂♂; II, 4 ♂♂; III, 3 ♀♀, 1 ♂; 20 = n , with every individual; temp. = 22-24°C.

α degrees	θ degrees		
	$A_{20} \alpha$	$A_{20} b$	$A_{20} c$
15		54.98 \pm 1.08	52.98 \pm 1.24
20	61.04 \pm 1.19	59.14 \pm 1.21	61.23 \pm 1.04
25	63.04 \pm 1.19	63.56 \pm 1.02	63.33 \pm 1.10
30	65.67 \pm 0.878	65.30 \pm 0.956	
35	70.25 \pm 0.724	69.02 \pm 0.741	67.67 \pm 0.857
40		73.17 \pm 0.739	
45	75.78 \pm 0.652		
50		76.79 \pm 0.588	76.46 \pm 0.607
55	78.28 \pm 0.607		
60			80.55 \pm 0.423
65		81.56 \pm 0.415	
70	79.05 \pm 0.607		80.07 \pm 0.410

TABLE II

Indices of variability for series of measurements of orientation of young rats of line A with distributed load. See text.

Series	V.N. $\sin \alpha$	Total variation	Percentage modifiable variation ($\alpha = 15-70^\circ$)	V.N.	Total variation	Percentage modifiable variation ($\alpha = 15-70^\circ$)	V.N. θ	Total variation	Percentage modifiable variation ($\alpha = 15-70^\circ$)
I	0.931	1.49	36.0	3.61	2.16	65.9	3.01	64.4	58.4
II	1.73	1.37	59.0	2.75	1.66	65.1	2.92	77.2	65.7
III	1.12	1.23	53.4	2.92	1.75	65.9	3.00	86.8	66.1

The total variation (P.E._g/ θ vs. $\log \sin \alpha$) averages 1.86 units, as contrasted with 1.3 units (corrected for N and for n) with unweighted

litters. Greater difficulty was experienced in attaching weights with precision in Series I. The weights varied a little more than in II, III; in Series I, 1.06 ± 0.015 gm., in II and in III, 1.05 ± 0.01 gm. was attached at each place.

The slope $\Delta P.E._\theta / (\Delta \sin \alpha = 0.4)$ varies a good deal (0.9 to 1.7) in the three series, but the total variation differs not so greatly

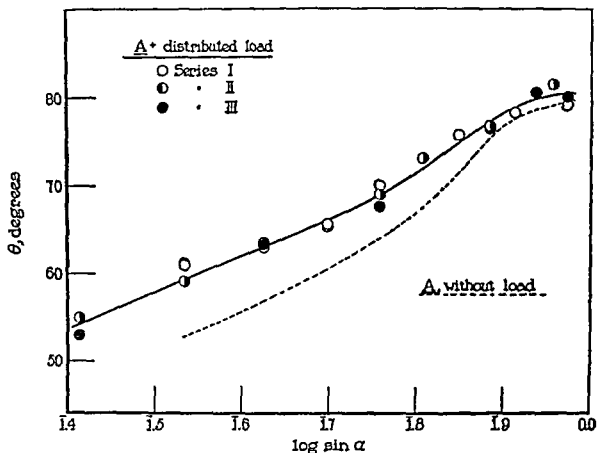


FIG. 1. Orientation-angle θ as related to slope of surface (α), for *A* rats with 1.06 gm. weight at shoulder level on the back with 1.06 gm. on back at level of the hips. Data in Table I. The dashed curve, for *A* rats without added load, is taken from Crozier and Pincus, 1931-32, *a*. The full curve is drawn without reference to the possibly significant "bump" at $\alpha = 20^\circ$. The analysis of this curve (in Fig. 9) is not disturbed in such a way as to alter the main conclusions at all if this possible irregularity is taken into account.

although the *percentage* of this total which is modifiable appears to be much lower in Series I than in II and III. $V.N._{\sin \alpha}$ is higher than with sets of unweighted *A* rats, as with $V.N.$ and $V.N._\theta$.

When $\Delta(100 P.E._\theta / \theta) / (\Delta \theta = 20^\circ)$ is computed (" $V.N._\theta$ ") it is quite constant (3.0) for the three series, its magnitude being a little

above that (2.3 to 2.8) for litters without weights, or with 2.1 gm. carried at shoulder level (Crozier and Pincus, 1931-32, *b*), but about equal to $V.N._\theta$ for animals with *posterior* load of 2.1 gm. ($V.N._\theta = 3.06$). The order of magnitude of this index of variability is essentially constant throughout, however. When comparison is made at equivalent orientation-angles (θ), the relative variation (Figs. 3, 4) and the absolute scatter of θ 's (Fig. 4) are practically independent of the presence of the added loads, when N and n are constant among

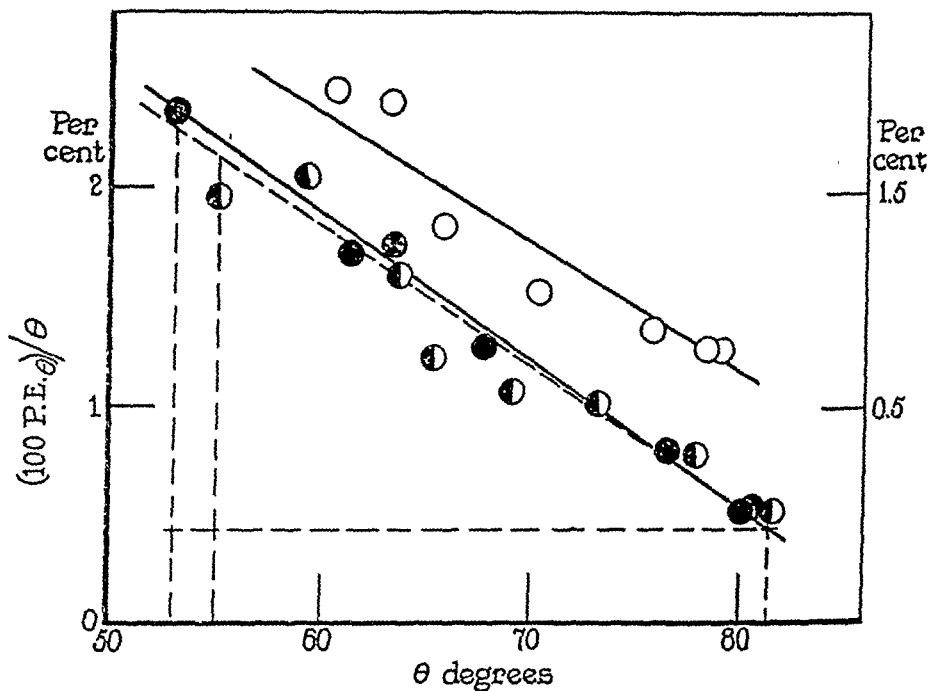


FIG. 2. The relative variation of orientation-angle as related to extent of orientation. The ordinate scale for Series I (open circlets) is at the right. See text.

the series compared. The reproducibility of the *variation* of θ is in this respect remarkable. It gives striking support to the view that θ is a measure of the excitation involved in determining orientation, and that the variation of orientation at constant slope of surface is governed by the intensity of the excitation, as we have already assumed (Crozier and Pincus, 1931-32, *b*, etc.). When the ordinary situation as regards the orientation has been altered, as by the imposition of added loads, this phenomenon of parallelism of θ and σ_θ is

quantitatively unaltered. This gives a proof that the correspondence of the two quantities, extent of orientation and its variation, is determined by an organic property of the race of rats whose performance is measured.

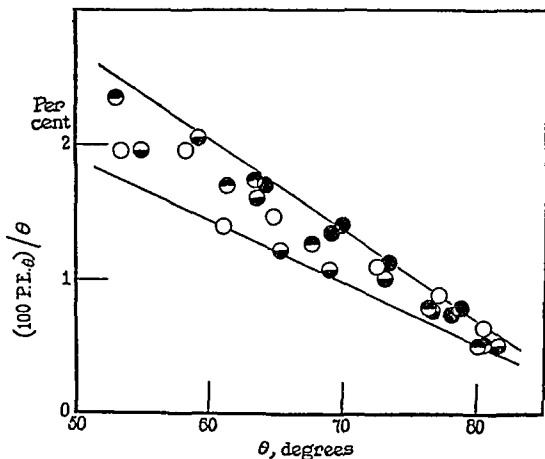


FIG. 3. $100 \text{ P.E.}_\theta/\theta$ vs. θ , for series of observations with A rats in which N , the number of individuals ($= 4$), and n , the number of observations (20) on each individual at each slope of surface, are identical:

- without added load (A_{18})
- with 2.12 gm. (A_{19}) posteriorly
- ◐ with 1.06 gm. anteriorly and 1.06 gm. posteriorly (A_{20} II)
- ◑ with " " " " " (A_{20} III).

The agreement of absolute magnitudes of relative variation of θ , at corresponding θ 's, is independent of the conditions of excitation as these are varied by altering the mechanical features of excitation; hence the dependence of variability of θ upon the magnitude of the response must be due to an organic interdependence. The variation of σ_θ with θ (Fig. 4) shows that this is no arithmetic accident. The slightly higher values of $\text{P.E.}_\theta/\theta$ for series A_{18} probably do not differ significantly from the others, as similar slight differences are encountered with "identical" series. Data for Series A_{18} , A_{19} , taken from previous papers (Crozier and Pincus, 1931-32, a) where the reason for a greater dispersion of σ_θ at the low- α end of the graphs is discussed. See Fig. 4.

The proportionate modifiable variation (65 *per cent*) is slightly higher than with unweighted *A* rats (56 *per cent*), the highest percentage with the unweighted series being 63. The figures are not significantly different for rats with anteriorly or with posteriorly carried loads. We might expect the percentage modifiable variation to be altered in one direction (decreased) by "errors" (differences of location) made in attaching the loads, and in the opposite direction

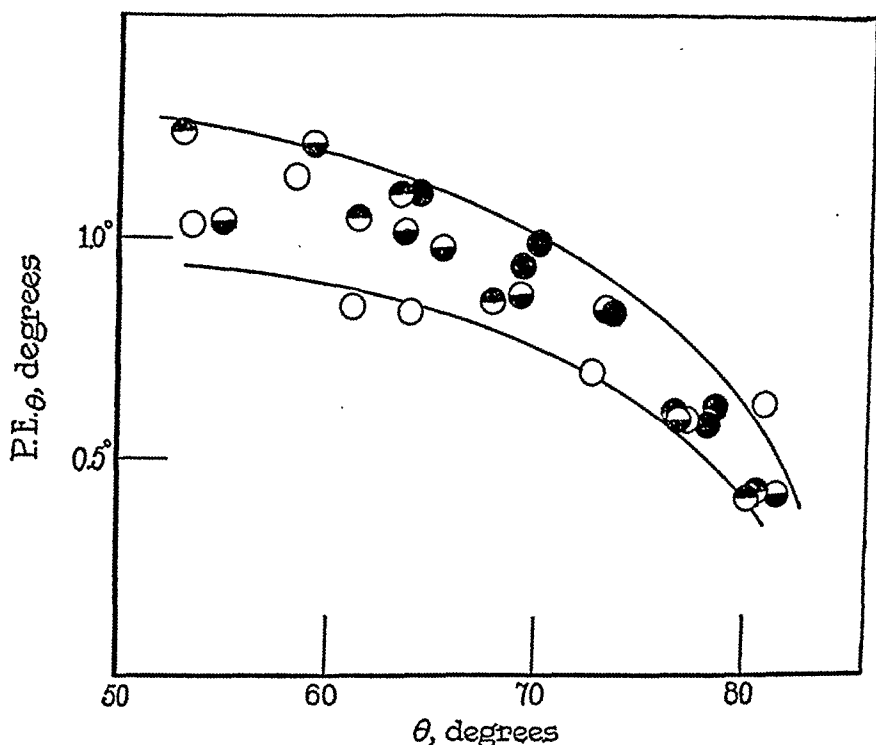


FIG. 4. The dependence of $P.E.\theta$ upon θ is independent of the presence of added loads transported during geotropic orientation, for series (different symbols) in which N and n are the same; see Fig. 3.

by the increased definiteness of excitation due merely to the augmented mass carried. Ideally, the latter effect is largely ruled out by comparisons made at equivalent θ 's (Figs. 2, 3). The fact that the proportionate modifiable variation is the same in the several series, as well as σ_θ when considered as a function of θ , demonstrates the absence of influential "errors" of purely technical kind, although this may account for the detectable increase of total observed variation. The slight increase of percentage modifiable variation is in

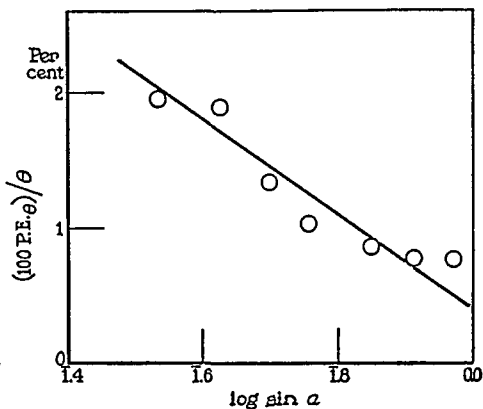


FIG. 5. $P.E._\theta/\theta$ vs. $\log \sin \alpha$, *A* rats with distributed load (2.1 gm.), Series I. See text, and Table II.

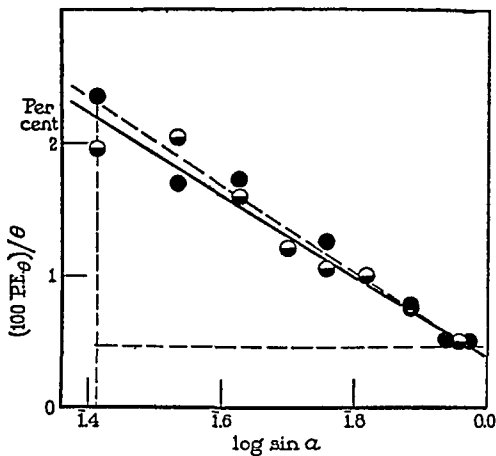
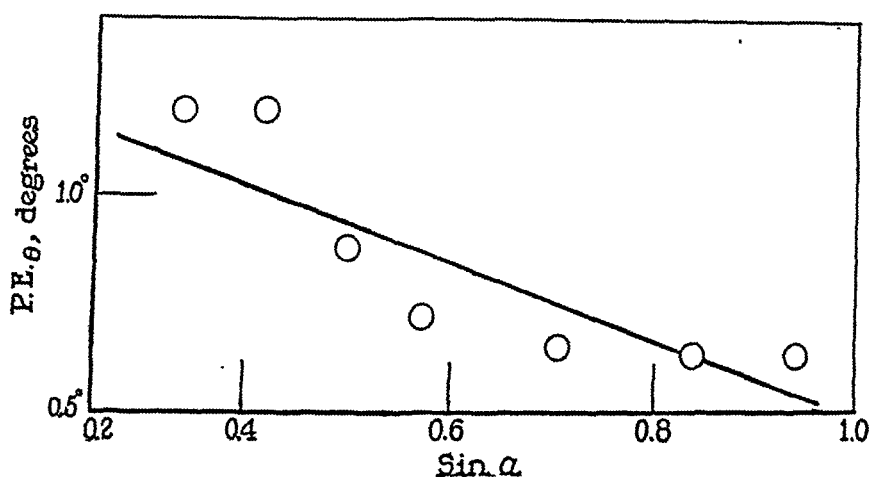
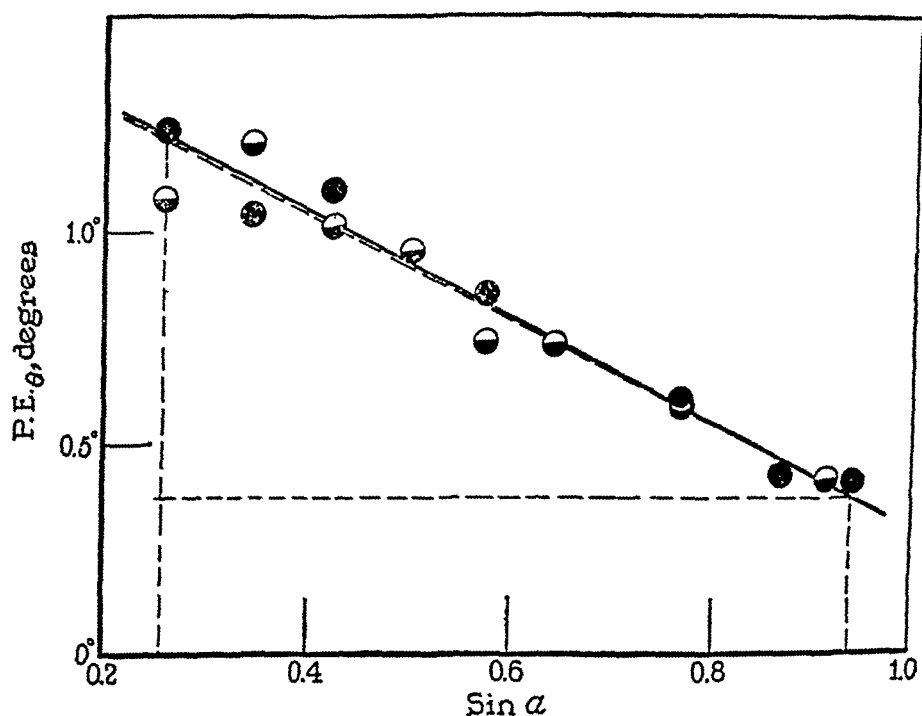


FIG. 6. $P.E._\theta/\theta$ vs. $\log \sin \alpha$, *A* rats (2.1 gm.), Series II, and III (solid circlets). See text and Table II.

FIG. 7. P.E._θ vs. sin α, Series I. (Cf. Table II)FIG. 8. P.E._θ vs. sin α, Series II and III. For each of these Series, $N = 4$, $n = 20$. The two sets are in close agreement (cf. Table II).

all likelihood due to the fact that the threshold α for orientation has been more precisely located ($\alpha = 15^\circ$) than in the case of the tests with unweighted *A* rats; correspondingly, in the series with *posterior*

load (Crozier and Pincus, 1931-32, *b*, p. 235), where percentage modifiable variation ($\alpha = 20-70^\circ$) was found "too low," it was certain that the real threshold for orientation was below $\alpha = 20^\circ$. The location of the effective threshold for unweighted rats at $\alpha = 18^\circ$ rather than at $\alpha = 20^\circ$ would bring the percentage modifiable variation for these to 65 *per cent*. To establish such small differences is a laborious matter, and the essential point can be approached more conveniently in other ways—notably by examining the form of the θ - α curve as a function of the age of the rats.

III

The basic assumption of our analysis has been that orientation is no longer constrained, during upward turning, when excitation becomes equivalent upon the two sides. With an added load "asymmetrically" disposed, that is, unbalanced as regards fore-and-aft distribution, the additional pull is localized, and a turning moment is introduced upon the inclined surface. If the added load is so arranged as nearly to eliminate such torques and differential pulls, we expect a general, non-selective action more or less equally exerted upon all three of our main "groups of receptors." The effect of this, when θ is plotted against $\log \sin \alpha$, and $\Delta_\theta/\Delta \log \sin \alpha$ against $\sin \alpha$, must be expected to exhibit itself as a general flattening of the curve θ *vs.* $\log \sin \alpha$, and of its derivative. Fig. 1 has already shown this; the kind of discontinuity evident when the same total added load concentrated at one posterior point is transported, is nowhere clearly apparent.¹ It becomes a very exacting test of the analysis already given (Crozier and Pincus, 1931-32, *a*, *b*) to see if for race *A* the total number of receptor units, and the numbers in each of the three recognizable subgroups of these, remain quantitatively unaltered when the *form* of the frequency distribution of their apparent thresholds is distorted, by carrying through the analysis without regard to the presence of the added weight and its effect upon the actual magnitude of the effective gravitational vector ("sin α "). The organic reality of these subgroups is already attested by their behavior in inheritance (Crozier and Pincus, 1929-30, *a*, *b*; and subsequent papers). Their nature as structurally distinct entities remains to be explored.

Fig. 9 gives $\Delta\theta/\Delta \log \sin \alpha$ from Fig. 1, plotted against $\sin \alpha$.

A curve has been fitted to the θ vs. $\log \sin \alpha$ plot (Fig. 1), assuming as usual that no minor discontinuities are to be recognized. The behavior of the variability functions shows that this is legitimate. Cases do arise in which this assumption cannot be made, and in these the dispersions of the measurements also exhibit discontinuity (*cf.* a subsequent report). The posteriorly located weight would be ex-

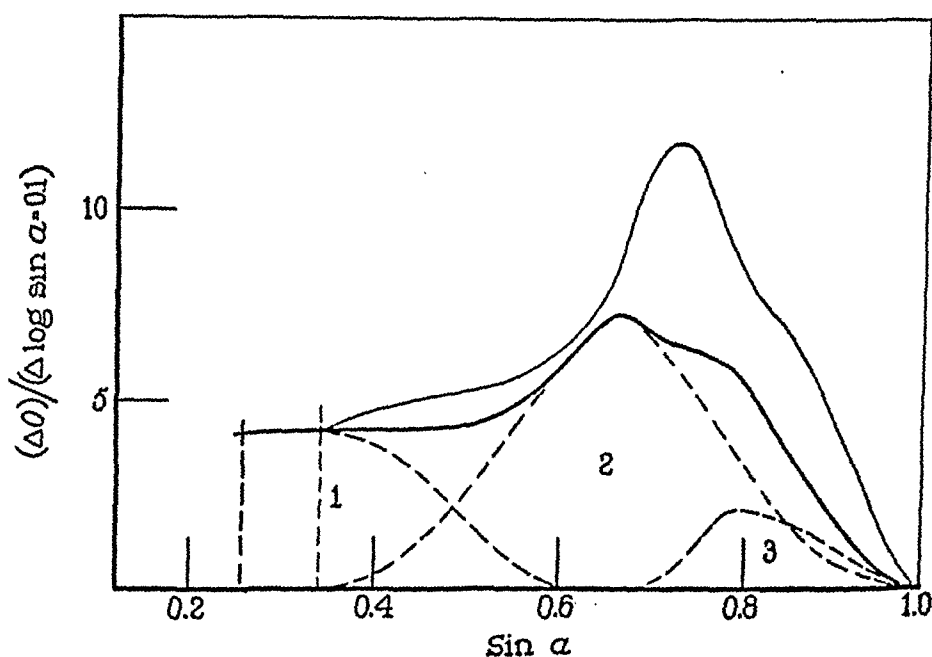


FIG. 9. $\Delta\theta/\Delta \log \sin \alpha$ vs. $\sin \alpha$, for A rats with distributed load (2.1 gm.), heavy line. Thin line gives corresponding distribution of receptor-unit thresholds for A rats without added load. See discussion in text. The curve for the loaded rats is resolved into three portions, which are in the text compared with the homologous regions 1, 2, 3, in the case of the unweighted individuals.

pected, of itself (*cf.* Crozier and Pincus, 1929-30, *b*; 1931-32, *b*), to raise a low- α segment of the curve, with decrease of its slope; the effect should not be so extreme with a 1 gm. weight as with 2 gm. (*cf.* Crozier and Pincus, 1926-27). The anterior weight should, by itself, slightly lower this end of the graph, and at intermediate inclinations cause it to ascend more steeply to a point above the curve for unweighted rats, though not so steeply nor so far above as with twice the additional mass. If the torque effects are practically

eliminated by the placing of the *two* weights, any averaging of the distortions of the θ - α curve due to the weights acting separately might or might not remove the flat intermediate portion appearing with posteriorly located weight alone. It seems to us unprofitable to stress the slight trace of flattening at $\alpha = 30^\circ$, although a case might be made out for it (*vide infra*).

If we differentiate the curve in Fig. 1, to obtain a picture of the relation of the effective distribution of the receptor thresholds involved, we must remember that the frequency of stepping is probably influenced in just the same way as θ by the added load, so that the proportionality factor in the relationship of $\Delta\theta/\Delta \log \sin \alpha$ to ΔR , the increment of activated tension receptors, would be expected to be different from that in the absence of the load. And if we plot $\Delta\theta/\Delta \log \sin \alpha$ against " $\sin \alpha$," the units of the latter must be recognized as made larger, since the gravitationally effective pull has been increased, at each slope of surface, by the presence of the added mass. What we want, however, is a picture of the disturbance of the "call" upon tension receptors due to the added mass as compared with the situation in its absence. This is secured by plotting $\Delta\theta/\Delta \log \sin \alpha$ against $\sin \alpha$, without correction for the additional mass, since we desire to see how this mass will bring sense organs into action at lower slopes than in the absence of the mass. We have assumed, with reason (Crozier and Pincus, 1929-30, *b*), that any increase $\Delta\theta$ in the excitation which θ measures will be dependent upon (1) an increase in the total number of receptor units (R) involved and (2) an increase in the frequency (F) of their excitation per gross unit of time, or:

$$\Delta\theta = K (\Delta R) (\Delta F);$$

since speed of progression was found to be a rectilinear function of $\log \sin \alpha$ (*cf.* Pincus, 1926-27),

$$\Delta\theta = K' (\Delta R) (\Delta \log g \sin \alpha)$$

where g is the added mass (since the weight of the individual is without significance). But $\Delta \log g = 0$; hence the original formulation is usable; K' will have a lower value with the added weight, however, since $\Delta F/\Delta \log \sin \alpha$ is less. In effect, the " $\sin \alpha$," without load, becomes $g \sin \alpha$. Hence in the graph of $\Delta\theta/\Delta \log \sin \alpha$ vs.

$\sin \alpha$ the ordinates (Fig. 9) must be multiplied by a constant, if the curve is to be compared with that when no weight is added. The area under the graph, in other words, will be less than it "ought" to be, because the scale in which ΔR is measured has been contracted. This difficulty can be gotten around, for all practical needs, by using ratios of the sub-areas into which the graphs for *A* rats weighted and unweighted may be resolved.

It is to be understood that several kinds of approximations are employed in any such derivation. Their justification appears in the coherence of the analysis and in the cogency of the end result when it is tested by independent criteria.

Fig. 9 gives the graph of $\Delta\theta/\theta \log \sin \alpha$ vs. $\sin \alpha$ for the *A* rats with "distributed load." The curve for unweighted *A* rats is also included (Crozier and Pincus, 1931-32, *b*). The area under the former is 8.23 units, under the latter 10.4 units. The curve for the weighted animals is resolvable into three portions, indicated in Fig. 9. The whole curve is flattened and pulled out to the left, as expected, and the maxima of its subcurves are moved to the left as compared with those for their homologues in the graph for unweighted individuals. The fact that Group 3 is deformed is consistent with the shift undergone by this group with anterior weight alone. The areas of the 3 subcurves ("Groups") are respectively:

Group	<i>A</i> rats	<i>A</i> + distributed weight	Ratios
1	3.10	2.43	1.27
2	6.18	4.94	1.25
3	1.12	0.86	1.30
Total.....	10.40	8.23	1.26

The ratios between these areas should be essentially constant, if the effect of the added load upon speed of progression is devoid of discontinuities. The only slight departure from constancy is with Group 3; the difficulty of fitting the upper limit of the curve is such that the slight departure is of no significance.

The highest θ attained is distinctly, though only slightly, above that measured without any added load (Fig. 1). This effect is

similar to that seen when an equivalent *anterior* load is carried (Crozier and Pincus, 1931-32, *b*); the explanation there suggested was, that the elevation of the centroid of the mass transported in creeping brought into play receptor units outside the usual tripartite distribution ("Groups 1, 2, 3"), although only to a slight extent. The difference previously encountered showed 1° additional in θ_{max} to be equivalent to 0.13 units of *R* area. The difference in θ_{max} is here 1° of arc, but the areal units are different, in the ratio unloaded:loaded::1.26:1. Hence if we subtract $(0.13)/(1.26)$ from 8.23 units, a better basis may be obtained for the comparison of the areas of the several groups of receptors in the two cases. That is, the area under the curve in Fig. 9 for the weighted rats is "too large" by the amount corresponding to a 1° increase in θ_{max} . If this increase is located in Group 2, its area should be diminished by 0.1 unit. The areal ratio for Group 2 then becomes $6.18/4.84 = 1.28$, and the corrected areal ratio for the complete curves $= 10.4/8.13 = 1.28$.

One apparent difficulty with this interpretation is found in the threshold θ . The threshold- θ supposedly signifies the least (mean) excitation capable of forcing orientation of progression in rats of a given race. It should be constant, unless central nervous thresholds are affected. The presence of an added weight brings about this amount of excitation at a lower inclination of surface than suffices in its absence. Since at low inclinations (α) excitation-units of Group 1 are alone implicated, the curve is "cut off" at the lowest θ measurable. Its completed form cannot be determined. Threshold θ measures the "missing" area under the curve; with and without weights these areas must be numerically identical, despite the implicit difference of ordinate scales. Hence the ratio of Areas 1 might not be significant. But since this ratio is the same as for Areas 2, it must be concluded that the "below threshold" excitation cannot be explored by this method; since there is no detectable orientation below threshold- θ , the frequency distribution of tension thresholds for this portion of the total array cannot be expressed in the units employed, and therefore the "missing" area appears as zero, in the coordinates used. The tension receptor units excited below threshold slope do not affect the orientation process at any intensity of

excitation. This is of course consistent with the parallelism between total area of receptor units and the index of modifiable variation (Crozier and Pincus, 1931-32, *b*).

SUMMARY

Young rats bearing a mass of 1.06 gm. both at shoulder level on the back and posteriorly at the sacrum exhibit in their geotropic progression a relationship between angle θ of oriented path and inclination α of substratum which differs from those obtained with rats of the same race carrying the same total added load concentrated anteriorly or posteriorly. The distributed load affects more or less equally all the groups of receptor units concerned in tension excitation. It is shown that the variation of orientation is organically determined, quantitatively, by the intensity of tension excitation, regardless of the imposition of the added loads. The bearing of these facts upon the theory of the geotropic orientation is discussed.

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ANALYSIS OF THE GEOTROPIC ORIENTATION OF YOUNG RATS. VI

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I

The result of crossing two well-inbred lines of organisms for each of which there has been established by repeated test a characteristic relationship between some aspect of performance or behavior and a measured external condition governing the performance, is important in at least two respects. It should contribute to the demonstration of the degree of reality inhering in the formulation used to express the characteristic relationship in point, and thus by implication at least to provide interpretation for it; constants in equations must be accorded physical significance if they can be shown to exhibit unitary behavior in inheritance. Conversely, the genetic utilization of such constants should provide rational, serviceable units of inheritance,—a means of really *defining* a gene¹ (*cf.* Crozier and Pincus, 1929–30, *a*). A first illustration of primitive steps in the technic of such experiments has been given in the treatment of the geotropic orientation of young rats (Crozier and Pincus, 1929–30 *a*; 1929–30, *b*; 1931–32, *a*, *b*). Essential additional features of such experiments (*cf.* also: Crozier, 1929; Crozier, Stier, and Pincus, 1929; Pincus, 1930–31; and Crozier and Pincus, 1931–32, *a*) are: the establishment of the connection between intensity of excitation, or of a condition controlling performance, and the measured response, for single individuals over a range of values of the experimental variable; the analytical description and utilization of the *variability* of performance; and the provision of

¹The relationship of this conception to certain current notions concerning “physiological” definitions of hereditary units has been referred to briefly in an earlier paper (Crozier and Pincus, 1929–30, *a*, p. 65); further development will be considered in another place.

independent experimental tests of the rationality of the equations used and of the basis for the constants involved.

Curves are established which exhibit for young rats the dependence of the angle of orientation (θ) during upward progression upon an inclined surface, upon the inclination (α) of the surface. For each pure line of rats investigated this curve proves to be a characteristic property, quantitatively recoverable in successive generations. So also are the measures of variability of orientation; the tests are so arranged that these measures can be employed to demonstrate the sensible absence of errors of observation. When two lines are crossed, for which the respective θ - α curves intersect at an intermediate slope of surface (Crozier and Pincus, 1929-30, *a*), the corresponding curve for the F_1 individuals was shown to be in a general way intermediate; the investigation of backcross generations gave clues to the real basis for this. A searching test of the notions implicit in and arising from such findings should be provided by the outcome of crossing another pair of lines, for which the respective θ - α curves do not intersect, but are roughly parallel and markedly separated on the θ - α grid. We have already indicated that in this latter instance the result, in F_1 , cannot be foretold completely from the results of the earlier experiment, but that we have every reason to look for different types of outcome in the two cases.

In crossing lines K and A , in the first experiment, the characterization of F_1 individuals involved conspicuously the slopes of the θ - α curve, which were superficially intermediate between those for the K and for the A rats. For races A and B differences in the slopes of the curves are not prominent, but their axis-intercepts are quite unlike. The data establishing the θ *vs.* α relationship for races A and B are given in an earlier paper (Crozier and Pincus, 1931-32, *a*). The fact that these two lines were originally segregated from the same loosely inbred stock should perhaps assist in the clarification of the experiment; the indications are very definite that commonality of origin determines persistent retention of at least some essential quantitative features of the geotropic response (*cf.* Crozier and Pincus, 1931-32, *a*, *b*). The ideas ordinarily exploited in genetic practice are not specifically useful in attempting to forecast the precise manner in which the F_1 individuals resulting from matings of A and B

rats should behave, either intrinsically or in contrast to F_1 individuals from the crossing of A and K strains. But it will readily be noticed that if from the raw data, analysis of the performance of the hybrid individuals and their descendants discloses the same fundamental units contributed by the A parents to the cross-bred individuals and their descendants, in the $A \times B$ experiment, as were recognizable in the test of $A \times K$, then a very powerful proof is given that the fundamental units in question are "real," although recognized by a somewhat complex procedure,—precisely in the sense that the properties of NaCl are independent of the previous histories of its two components. For in the event that the experiment is to this extent unequivocal and successful, it will amount to giving demonstration that constants in the descriptive equations can be recovered unchanged after submergence (as "recessives") in two quite different genetic complexes.

The results of crossing races A and B are given in this and following papers. We discuss first the behavior of F_1 individuals, in some detail, and illustrate criteria for the classification of individuals. Details of observational technic are recorded in contributions already cited. A complication in the data for F_1 is satisfactorily resolved by the behavior of individuals produced by mating F_1 with rats of parent race B . This complication, however, is of such a nature that it becomes necessary to consider certain problems of "heterosis" and "hybrid vigor" from a new standpoint.

II

Five litters from matings of A and B rats were examined, a total of twenty-two individuals. The history of these matings is given in Table I.² We may first examine the mass properties of the several litters, before considering the effects of grouping together data from individuals produced in different litters of the same ancestry. The

² In a paper continuing his analysis of the somewhat similar but by no means identical problem of facet-number vs. temperature in *Drosophila*, Hirsh (1930, p. 293) states that in our crosses of races K and A we failed to give data for reciprocal matings. This is not quite correct; in our paper (Crozier and Pincus, 1929-30, α , b) data on offspring from reciprocal matings were not separated, since they showed no differences.

latter procedure is desirable when arriving at the interpretation of the behavior of the rats in subsequent generations.

The observations are summarized in Table II. The parent rats

TABLE I

Five matings producing litters examined for geotropic response in hybrids of the *A* and *B* lines; 13 days; temp. 23.5°C.

No.	Mating	Number in litter <i>N</i>	Weight <i>gm.</i>
<i>F</i> ₁ I	<i>B</i> ₁₇ ♀ / <i>A</i> ₁₇ ♂	5 (3♂, 2♀)	17.2±1.5
II	<i>B</i> ₁₇ ♀ / <i>A</i> ₁₇ ♂	5 (3♂, 2♀)	
III	<i>A</i> ₁₈ ♀ / <i>B</i> ₁₇ ♂	3 (3♂)	15.8±0.8
IV	<i>A</i> ₁₈ ♀ / <i>B</i> ₁₇ ♂	4 (3♂, 1♀)	16.4±0.9
V	<i>A</i> ₁₈ ♀ / <i>B</i> * ₁₇ ♂	5 (3♂, 2♀)	18.2±0.3

* Father of the litter used for Series *B*₁₈II (Crozier and Pincus, 1931-32, *a*).

TABLE II

Mean angles of geotropic orientation (θ) for young rats of the hybrid generation produced by crossing rats of the *A* and *B* lines; $n = 20$ for each individual at each slope (α); N = number of individuals.

α , degrees	θ , degrees				
	Litter <i>F</i> ₁ I <i>N</i> = 5	<i>F</i> ₁ II <i>N</i> = 5	<i>F</i> ₁ III <i>N</i> = 3	<i>F</i> ₁ IV <i>N</i> = 4	<i>F</i> ₁ V <i>N</i> = 5
15	58.02±0.977	55.70±0.679	56.38±1.11		56.03±0.891
20	60.48±0.775	60.40±0.796	62.11±1.01	62.48±0.619	
25	67.96±0.625	65.42±0.656		65.57±0.702	64.60±0.755
30	67.02±0.678	65.51±0.584	68.59±0.993	70.98±0.804	67.86±0.901
35	74.56±0.591		72.89±0.660	73.05±0.584	72.26±0.567
40		73.98±0.498		75.11±0.751	74.38±0.527
45	76.39±0.505		76.78±0.562	77.85±0.479	76.73±0.591
50		80.00±0.488			78.99±0.548
55	81.44±0.424		81.03±0.532		
60	81.63±0.434			82.73±0.381	
70	82.96±0.387	82.80±0.342	83.09±0.494		83.15±0.353

had themselves been tested, or their sibs. Rats of Litter IV were relatively weak; the mother did not nurse well. The data are given graphically in Fig. 1.

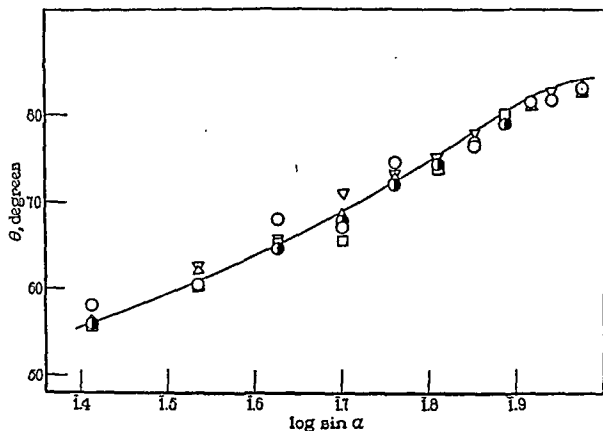


FIG. 1. Mean angles of oriented path (θ), as a function of inclination of substratum (α), for five litters of hybrid offspring produced from matings of rats of lines A and B; see Table II. The mean curve for B rats is given by the solid line. Above $\alpha = 35^\circ$ the observations consistently lie below the curve for the B parent line; at lower inclinations the general weight of the observations is above the B curve, and the latitude of variation is much greater.

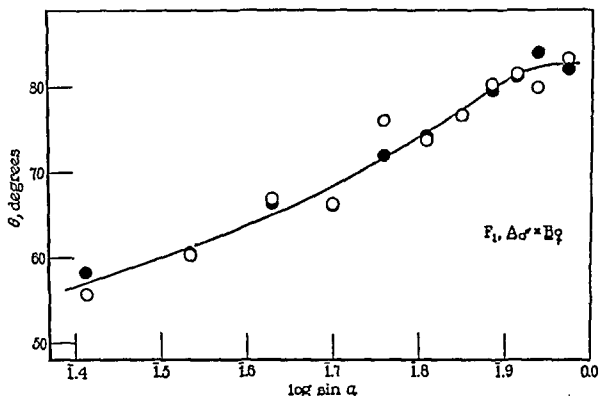


FIG. 2. The relation of average orientation — angle θ to slope of surface (α) is practically the same for $\sigma\sigma$ (open circles) and $\varphi\varphi$ from the matings A σ \times B φ (Litters I and II, Table III); P. E. σ_m 's for the several averages overlap, except at $\alpha = 35^\circ$, the "transition region" already noted (Fig. 1).

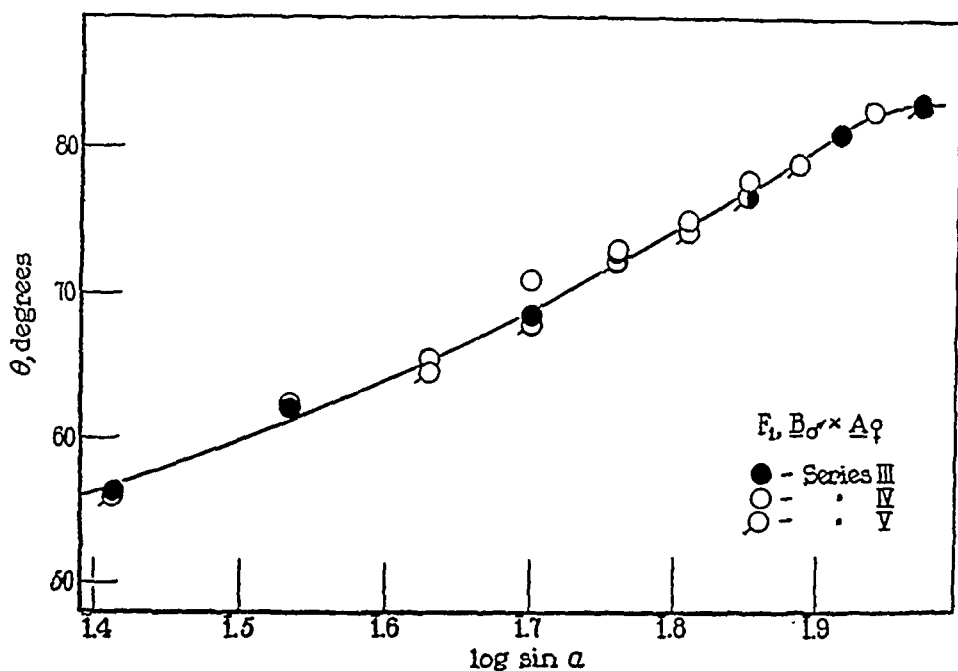


FIG. 3. θ vs. $\log \sin \alpha$ for ($B\sigma \times A\phi$) litters. The latitude of scatter of θ_m is less than with ($A\sigma \times B\phi$), below $\alpha = 35^\circ$. (Cf. Fig. 4.)

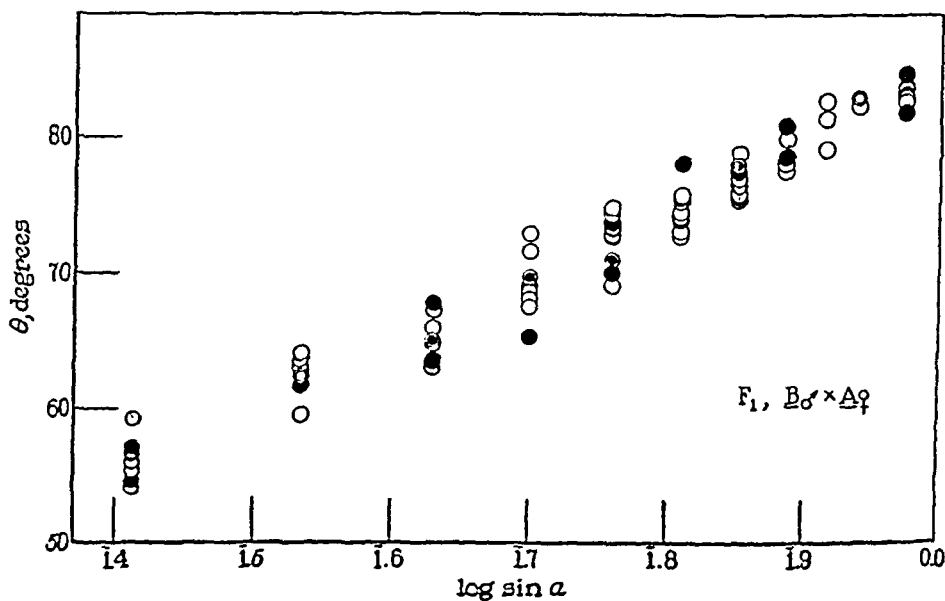


FIG. 4. θ vs. $\log \sin \alpha$, for σ and ϕ individuals, from matings ($B\sigma \times A\phi$); cf. Fig. 3; $\phi\phi$ as solid circlets.

In a very general way the concordance of the results from the four litters is satisfactory, although at $\alpha = 30^\circ$ the divergences are perhaps not covered adequately by the standard deviations of the mean θ 's. One observation (Litter I, $\alpha = 25^\circ$) is definitely "off," θ being too high; it is noteworthy that P.E._g is here correspondingly "too low"—a fact noticeable in a few other cases as well, and traceable to the intimate connection between the magnitude of mean θ and its variation (Crozier and Pincus, 1931-32, *a*, *c*). Before attempting to consider the observations more analytically it is desirable to see how their general properties compare with those for the corresponding curves of the parent stocks. It is apparent at once that with respect to (1) threshold for orientation, (2) maximum θ attained, and (3) shape of curve, including position of its inflection, the F_1 families are very similar, and are closely comparable with the B parents. So close is this comparison (Fig. 1) that one might be tempted to consider the state of affairs one of simple dominance. To this extent the expectation we had been led to form, that F_1 individuals from the crossing of A with B should show a relation to the parent strains different from that discovered in the hybrids of A with K , is substantiated in a rather striking way.

III

The consistency of the records obtained from the several litters, with respect to the differences their θ - α curves exhibit when compared with those for the B line, however, are suggestive and inviting. It is easily noted that up to $\alpha = 30^\circ$ the weight of the observations is definitely *above* the B curve; that an unusual degree of scatter is apparent at $\alpha = 30^\circ$, with some suggestion of discontinuity in the curve; and that above $\alpha = 35^\circ$ the latitude of variation along the $\log \sin \alpha$ axis is abruptly and consistently reduced to about one-third its value below that slope (*cf.* Crozier and Pincus, 1931-32, *a*). The uppermost portion of the curve, with narrowed fluctuation, is slightly but continuously below the B curve.

Our previous experience with a good number of litters leads us to believe that these peculiarities are probably significant.

Litter III consisted of males exclusively, and its performance adheres most closely to that of the B parents,—in fact could not by

TABLE III

Orientation-angles for F_1 individuals from matings of A and B rats ($A\sigma \times B\varphi$); $n = 20$

		$\alpha = 15^\circ$	20°	25°	30°	35°	40°	45°	50°	55°	60°	70°
Orientation-angles for F_1 individuals from matings of A and B rats ($A\sigma \times B\varphi$); $n = 20$												
No.	Litter	$\sigma \sigma$										
1	I	58.7±2.34	60.7±2.17	67.3±1.40	67.2±1.87	75.0±1.59		74.7±1.36		81.0±0.936	78.8±1.25	84.4±0.846
2	"	55.0±3.57	61.3±1.80	70.7±1.44	66.6±1.52	76.8±1.44		76.9±1.47		81.3±1.00	80.4±1.19	82.8±0.886
3	"	56.3±2.26	59.4±1.97	65.9±1.35	67.4±1.68	76.6±1.40	72.7±1.39	77.2±1.17		81.3±1.00	80.4±1.19	84.3±0.941
7	II	54.8±1.77	55.8±2.52	67.5±1.37	65.3±1.26		74.6±1.04		80.4±1.43	82.6±0.694	80.7±1.07	82.0±0.882
8	"	54.6±1.55	62.0±1.73	64.6±1.75	66.4±1.42		73.9±1.20		80.2±0.902			85.1±0.698
9	"	56.5±1.10	63.2±2.38	66.2±2.16	65.1±1.26				80.3±1.22			82.1±1.11
$\varphi \varphi$												
4	I	58.5±1.61	61.6±1.64	68.0±2.14	66.7±1.32	71.8±1.34		76.1±1.20		79.4±1.03	83.4±1.03	79.95±1.11
5	"	62.0±1.79	59.5±1.79	68.4±1.18	67.2±1.80	72.4±1.38		76.9±0.785		83.2±0.894	84.6±0.767	83.6±0.906
6	II	56.6±1.81	61.2±1.27	65.2±1.16	65.2±1.43		72.7±1.18		80.0±1.28			83.7±0.581
10	"	55.9±2.01	60.2±1.74	63.7±1.53	65.6±1.73		75.9±1.25		78.7±1.11			81.5±0.872

itself really be distinguished therefrom by any reasonable test. Occasionally, a rather similarly divergent litter is encountered in a pure strain (*cf.* Series B'I, in: Crozier and Pincus, 1931-32, *a*). To determine if sex-linked recessive genes introduced from one stock may appear to be involved in this matter we must carefully examine the behavior of the two sexes in the reciprocal crosses. One is encouraged to do so by the behavior of the indices of variability, although in our previous experiments with *K* and *A* lines no differences of this sort were detectable. The effect sought might be one involving in part the *variation* of θ (*cf.* Fig. 1), which could complicate the picture very considerably. In this connection a distinction must be made between the "variability number" (V.N.) and the percentage of the total observable variation of θ which is modifiable as a function of the intensity of excitation (*cf.* Crozier and Pincus, 1931-32, *a*). In hybrids of *K* and *A* both measures of variability are low—a reduced proportion of the variation of θ is controllable as a function of the intensity of gravitationally induced excitation, by comparison with the parent stocks. The point we have to examine is the possibility that mean θ and its variation (σ_θ) may be correlated with the sex of the individual, or with some other detectable feature of organization with respect to which our total F_1 population may be heterogeneous. The present method of analysis, however, has the important advantage that we are able to examine the *variability of performance* of each individual separately, thus obtaining an independent index of any "heterosis" phenomenon which the measurements of behavior may reveal.

The data for $\sigma\sigma$ and for $\varphi\varphi$ are given individually in Tables III and IV. The irregularity or discontinuity of the general curve (band) at $\alpha = 35^\circ$ (Fig. 1) is perfectly real, as is also the reduced horizontal extent of the band above $\alpha = 35^\circ$. Mean θ for all F_1 $\sigma\sigma$ is identical with mean θ for all F_1 $\varphi\varphi$. The behavior of the rats produced in the reciprocal matings is not entirely uniform, however. Between the $\sigma\sigma$ offspring of $A\sigma \times B\varphi$ and $B\sigma \times A\varphi$ there are no systematic, ordered differences in mean θ ; but $\varphi\varphi$ from $A\sigma \times B\varphi$ tend to give slightly lower mean θ 's throughout than do $\varphi\varphi$ from $B\sigma \times A\varphi$. In the $A\sigma \times B\varphi$ cross, $\sigma\sigma$ and $\varphi\varphi$ are quite indistinguishable (Fig. 2), whereas for the cross $B\sigma \times A\varphi$ there is perhaps

TABLE IV
Orientation-angles for F₁ individuals from matings of A and B rats (B ♂ × A ♀); n = 20

N ₀	Litter	α = 15°	20°	25°	30°	35°	40°	45°	50°	55°	60°	70°
$\sigma^1 \sigma^1$												
11	III	56.0±2.01	64.1±1.61		68.6±1.71	71.0±1.08		76.5±0.898		82.7±0.892		83.1±1.03
12	"	56.6±2.23	59.5±2.08		68.1±2.00	72.7±1.27		78.0±0.921		79.2±1.28		83.5±0.604
13	"	56.6±1.87	63.0±1.68		69.0±1.69	74.8±1.13		75.9±1.32		81.4±0.670		82.6±1.01
14	IV		62.4±1.25	64.8±1.46	72.9±1.47	74.4±1.33	72.2±1.86	78.0±1.04			82.8±1.02	
15	"		62.4±1.57	67.3±1.51	71.6±1.82	69.1±1.31	75.8±1.56	77.0±1.29			82.3±0.851	
16	"		63.6±0.989	65.4±1.70	69.7±1.94	73.4±1.09	74.5±1.57	78.9±0.847			82.9±0.471	
21	V	55.4±2.10		66.0±2.10	68.8±2.39	72.8±1.17	74.2±0.950	77.9±1.18	79.9±1.37			83.0±0.863
22	"	54.1±2.40		63.3±1.61	68.3±2.40	74.5±1.26	73.1±1.40	75.4±1.32	77.6±1.12			83.6±0.737
24	"	59.2±1.92		63.0±2.14	67.5±1.69	73.3±1.59	75.3±1.20	76.4±1.55	78.1±1.45			82.8±0.869
$\sigma^1 \sigma^1$												
$\sigma^1 \sigma^1$												
17	IV		61.7±1.61	65.0±1.45	69.5±1.75	73.7±1.17	78.1±1.49	77.5±1.03			82.9±0.776	
20	V	57.1±2.17		63.5±1.64	65.3±2.24	70.0±1.64	73.9±1.74	78.0±1.27	78.6±1.55			81.8±1.05
23	"	54.6±2.20		67.8±1.65	69.7±2.26	71.0±1.19	75.5±1.10	75.6±1.81	80.9±1.18			84.6±0.766

a detectable difference between $\sigma^7\sigma^7$ and $\varphi\varphi$ progeny (Figs. 3, 4). The number of individuals is small, and the differences in θ might be regarded as altogether too slight for significance but for the consistency of individuals. With $B\sigma^7 \times A\varphi$ rats (Fig. 3) the mean θ -curve is almost indistinguishable from that for the B parents, up to $\alpha = 40^\circ$; above that slope the graph passes consistently below that for B , and, as with the $A\sigma^7 \times B\varphi$ rats, the horizontal latitude of variation is sharply diminished. The difference between the offspring in the reciprocal matings is summarized in the statement that below $\alpha = 35^\circ$, the (increased) latitude of scatter of θ is greater with the ($A\sigma^7 \times B\varphi$) rats. In the light of the consistencies of performance already demonstrated among pure-line individuals (and in the subsequent generation of the present experiment), the peculiarities of these data call for explanation. If concordant indications are obtainable from the indices of variability of θ , the case for the reality of these peculiar features can of course be strengthened.

IV

The indices of variation of θ as a function of α (*cf.* Crozier and Pincus, 1931-32, *a, b*) are given in Table V. They show that the *variability* of orientation-angle is *about* the same for the two sexes in each cross, but that the dependence of σ_θ upon $\sin \alpha$ is greater for $B\sigma^7 \times A\varphi$ individuals, than for the progeny of the reciprocal mating (Figs. 5-14). This difference is uniformly apparent when the figures for single individuals are used, as well as in the massed data. It cannot be traced directly to the arithmetic influence of the magnitudes of mean θ , because the graphs of $\Delta P.E._\theta / \Delta \sin \alpha$ show quantitatively similar contrasts. The difference is clearly consistent with the behavior of the θ_m - α curves already discussed; and it is thus of interest for the view (Crozier and Pincus, 1931-32, *c*, etc.) that θ_m and its variation are organically interconnected.

In Table V we have included indices of variation for lines A and B . For several reasons the instructive comparisons are with the figures for B , notably because the threshold α for A is 20° , whereas it is 15° for B and for F_1 ; this prevents direct comparisons of percentages of modifiable variation for A . It is clear that while $\Delta P.E._\theta / \Delta \sin \alpha$ is about the same in the F_1 groups as in B rats, the

factor $\Delta(P.E._g/\theta)/\Delta \log \sin \alpha$ is definitely *lower*, on the average, although not markedly so. The percentage of the total variation which is modifiable is significantly reduced, however, in both $\sigma\sigma$ and $\varphi\varphi$ of F_1 , whether from $A\sigma \times B\varphi$ matings or from $B\sigma \times A\varphi$. Both sexes have slightly higher relative modifiable variation of θ in the case of the $B\sigma \times A\varphi$ matings. The slightly higher modifiable variation for $\sigma\sigma$ as compared with $\varphi\varphi$, in the case of both matings,

TABLE V

Indices for variation for F_1 progeny from matings of A rats with B

		$\sigma^{\delta} \sigma^{\delta}$	$\varphi \varphi$
A	V.N. _{log sin α}	2.82	3.32
	V.N. _{θ}	2.56	2.40
	Modifiable variation 56 <i>per cent</i>		74 <i>per cent</i>
	V.N. _{sin α}	0.93	1.11
		$\sigma^{\delta} \sigma^{\delta}$	$\varphi \varphi$
A $\sigma^{\delta} \times B \varphi$	V.N. _{log sin α}	2.58	3.54
	Modifiable variation 53.6 <i>per cent</i> (by litters)		52.5 <i>per cent</i>
	Total variation 1.87 (by litters)		2.67
	V.N. _{θ}	2.72	3.48
	Modifiable variation 57.1 <i>per cent</i>		50.2 <i>per cent</i>
	V.N. _{sin α}	1.03	1.60
B $\sigma^{\delta} \times A \varphi$	V.N. _{log sin α}	3.1	3.03
	Modifiable variation 62.1 <i>per cent</i>		59.6 <i>per cent</i>
	Total variation 1.95		3.26
	V.N. _{θ}	2.92	2.03
	Modifiable variation 57.9 <i>per cent</i>		60.9 <i>per cent</i>
	V.N. _{sin α}	1.3—	1.0

is scarcely significant. The general result must mean that there is present in the data for the F_1 population a decrease in the *proportion* of total variation of performance which is open to control by the condition eliciting the performance. But this does not necessarily signify merely that the total variation has been increased by hybridization. The areas under the variation plots, with ordinates corrected for size and constitution of sample, are distributed in such a way

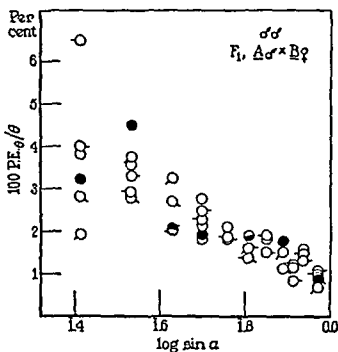


FIG. 5

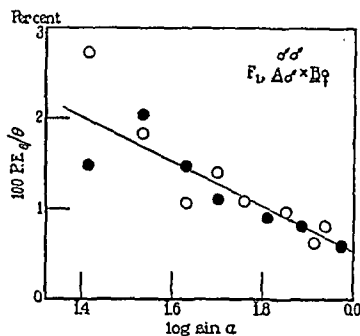


FIG. 6

FIG. 5. 100 P.E.g/θ vs. log sin α, individual ♂♂ from matings A ♂ × B ♀. Average V.N. = 2.20, modifiable variation = 63.3 per cent.

FIG. 6. 100 P.E.g/θ vs. log sin α, ♂♂ by litters: open circles, Litter I (N = 3); solid circles, Litter II (N = 3). These comparable sets agree well; V.N. = 2.58, modifiable variation = 53.6 per cent (total variation = 1.87 units).

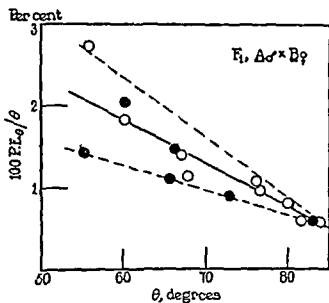


FIG. 7

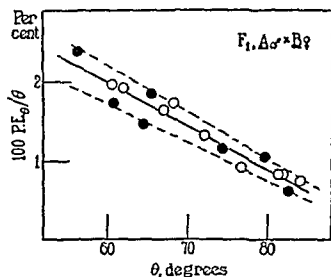


FIG. 8

FIG. 7. 100 P.E.g/θ_m vs. θ, for ♂♂, A ♂ × B ♀, by litters: open circles, Litter I (N = 3); closed circles, Litter II (N = 3). V.N._g = 2.72, modifiable variation = 57.1 per cent.

FIG. 8. 100 P.E.g/θ_m vs. θ, for ♀♀, A ♂ × B ♀; open circles Litter I (N = 2); closed circles, Litter II (N = 2). V.N._g = 3.48; modifiable variation = 50.2 per cent.

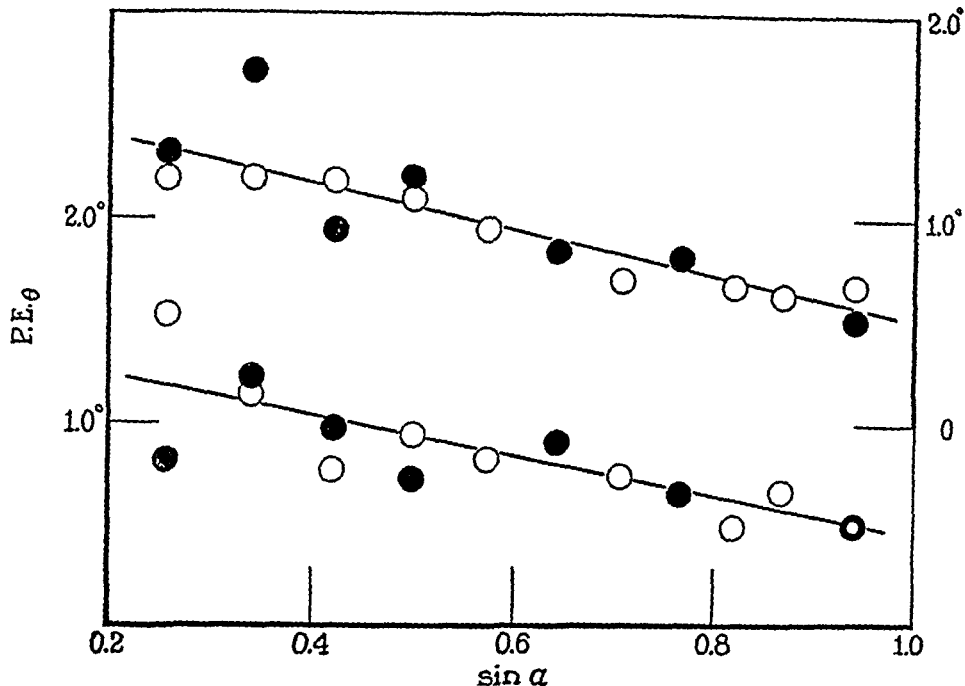


FIG. 9. $P.E.\theta$ vs. $\sin \alpha$, F_1 ($A \sigma \times B \varphi$). Upper plot, ♀♀, Litter I ($N = 2$), open circles; Litter II ($N = 2$), solid circles. Lower plot, ♂♂, Litter I ($N = 3$), open circles; Litter II ($N = 3$), solid circles.

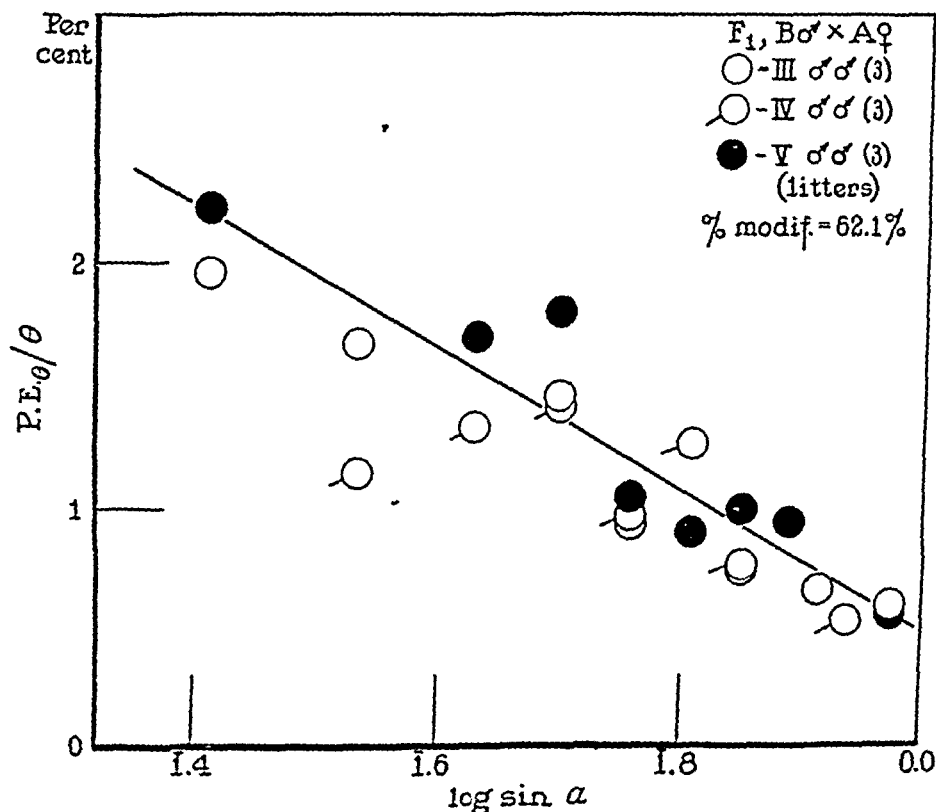


FIG. 10. $100 P.E.\theta/\theta$ vs. $\log \sin \alpha$, F_1 ($B \sigma \times A \varphi$), by litters; $N = 3$ in each case; modifiable variability = 62.1 per cent.

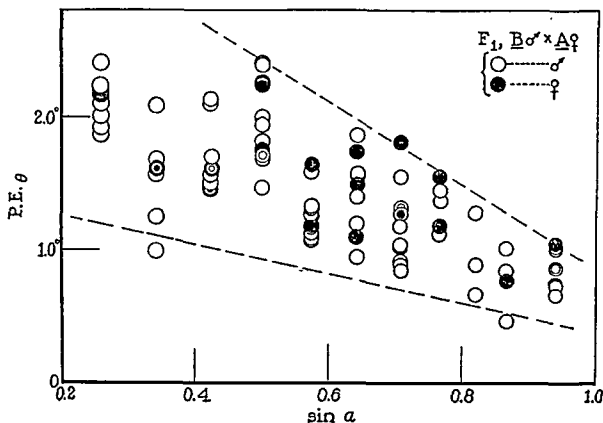


FIG. 11. $P.E.\theta$ vs. $\sin \alpha$, $F_1 (B\sigma \times A\varphi)$, individually; open circlets, data from $\sigma\sigma$; solid circlets, data from $\varphi\varphi$.

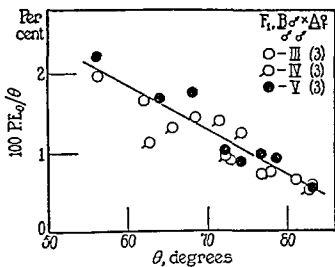


FIG. 12

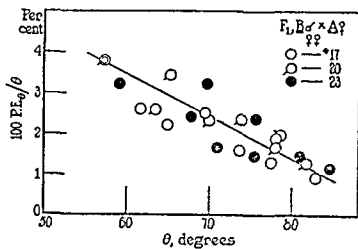


FIG. 13

FIG. 12. $100 P.E.\theta/\theta$ vs. θ , $F_1 (B\sigma \times A\varphi)$, $\sigma\sigma$ from Litters III (open circles), IV (circlet with tag), V (solid circles).

FIG. 13. $100 P.E.\theta/\theta$ vs. θ , $F_1 (B\sigma \times A\varphi)$, $\varphi\varphi$ individuals. V.N. = 2.03, modifiable variability = 60.9 per cent.

as to show that the total variation ($P.E._\theta/\theta$ vs. $\log \sin \alpha$) is not significantly different in F_1 from that apparent in B : for F_1 the mean total variation is 1.6, that for B is 1.5, that for A , 1.3 units. With A the threshold is at $\alpha = 20^\circ$; if the B plots be cut off at this slope, the total variation is of course less (Crozier and Pincus, 1931-32, *a*). This clearly demonstrates that it is the "uncontrollable" variation of θ which is increased in F_1 .

From the standpoint of modifiable variation of θ , $\sigma\sigma$ from the two crosses are identical, while $\varphi\varphi$ from $A\sigma \times B\varphi$ exhibit a *lower* modifiable variation than do those from $B\sigma \times A\varphi$; in the cross $B\sigma \times A\varphi$ the two sexes are indistinguishable from this standpoint. We have already noted that $\varphi\varphi$ from $A\sigma \times B\varphi$ tend to give slightly lower mean θ 's throughout than do the $\varphi\varphi$ from $B\sigma \times A\varphi$, whereas for $A\sigma \times B\varphi$ the sexes are indistinguishable. It is difficult to avoid the conclusion that this parallelism in the behavior of θ_m and of σ_θ must be indicative of a very intimate connection between the two measures of performance. Nor can we refrain from pointing to the singular and encouraging fact that the slight differences between the young rats, according to sex and to the type of mating producing them, are identically revealed in the behavior of θ and of σ_θ , so that knowledge of these differences could be obtained from the *variation* of performance alone.

It is important to note that the heterogeneity of the F_1 population, as revealed in the foregoing discussion, is also substantiated in another way. If indices of variability are computed on the basis of mean θ 's for a whole *litter* of F_1 rats, lumping data for $\sigma\sigma$ and $\varphi\varphi$, the variability numbers come out much too low (Figs. 9, 14) and the percentage modifiable variation is inordinately reduced.

Clearly, it is difficult to account for a difference between $\varphi\varphi$ progeny in reciprocal crosses on simple genetic grounds. Nor are we required, in fact, to do more at the moment than describe the properties of the individuals concerned in our particular experiment. Evidence is subsequently considered which leads to the opinion that the differences in the *shape* of the θ - α curve are of a purely developmental, rather than genetic, type, and that they are not necessarily connected with the phenomena of "heterosis" apparent in the magnitudes of the indices of variation by comparison with that for the B

race. The mean indices of variation for the *A* and *B* parental lines (*cf.* previous papers) are:

Race	<i>B</i>	<i>A</i>
V.N.....	3.32	2.82
V.N. _g	2.40	2.56
Modifiable var.....	74 per cent	56 per cent
V.N. _{sin α}	1.11	0.93

The measures of $\Delta P.E._g / \Delta \sin \alpha$ are slightly higher for *B* individuals than for *A*, and in the *F*₁ individuals the order of magnitude is pretty clearly that for the *B* type (*cf.* Table V). This is also true for V.N. and for V.N._g. The percentage modifiable variation, however, is definitely below that for *B*, by an amount which is quite significant. It falls in with the magnitude obtained for *A* rats, but it is to be recalled that the latter have a higher threshold α ($= 20^\circ$) for orientation than *B* or *F*₁ ($\approx 15^\circ$). The lowered proportionate modifiable variation is of course in keeping with the increased latitude of scatter of θ (below $\alpha = 35^\circ$); it would be only slightly increased if, as is likely, the real threshold for the *F*₁ rats is a little below $\alpha = 15^\circ$. We have already pointed out that the *total* variation of performance is only slightly increased, in *F*₁, and that the horizontal latitude of scatter in graphs of θ *vs.* $\log \sin \alpha$ is a measure of the uncontrolled variation of orientation (Crozier and Pincus, 1931-32, *a*). If these relations are to be interpreted as in any degree due to the action of independently assorting factors which secondarily affect the modifiable proportion of variation, then their presence must continue to be manifest in further generations of the rats derived from *F*₁. We shall have occasion to see that this is indeed the case. The influences in question might or might not be associated with the general "lifting" of the lower portion of the θ - α curve (Fig. 1), by comparison with that for *B*, and the "dropping" of its upper segment. Evidence considered later in this paper indicates that they must be regarded as unconnected.

V

Situations probably not unlike the present one are not infrequently attributed to "heterosis." The common experience that in *F*₁ the

"variability" of the population is decreased must be subject to further analysis before it can be fruitfully interpreted. In the present instance it is clear that the indices of variation (V.N., etc.) are closely similar to those for one parent, and that the total variation of performance is certainly not decreased; the proportion of "controllable variation" is definitely decreased, but this is quite a different thing. The method of establishing properties of a genetically homogeneous population as a function of one or more independent variables should be productive in this respect. If the present case be taken as suggestive, it is clear that new sorts of complexity are revealed as possible in such phenomena. It is not adequate to refer merely to the absolute or relative magnitudes of dispersion of measured properties, since these quantities may be demonstrably functions of other variables; in general it is to be presumed that they are. Moreover, where (as we have earlier suggested: Crozier and Pincus, 1929-30, *b*) a "multiple factor" effect is in question, the influence labelled "heterosis" for convenience in the present case may be expressed in contrary ways under different external conditions. At low slopes of surface the horizontal scatter of θ_1 (Figs. 1, 2) is increased, at high slopes decreased, by comparison with the curves for the dominant parent. Yet—"contrary" to the uniformly encountered *inverse* relation between θ and σ_θ (within a genetically homogeneous group of individuals),—we find that θ_1 is uniformly *greater* than for *B*, at slopes of surface $< \alpha = 40^\circ$, though *less* than for *B* above $\alpha = 40^\circ$. If the lower portion of the curve represents "hybrid vigor,"—and it is legitimately compared with the sorts of phenomena so labelled—to what does its upper portion correspond? Clearly, some further analysis is called for. We believe that precisely similar considerations must ultimately be invoked in the treatment of other examples of "heterosis," notably in connection with growth. This must involve not only the discussion of equations relating to aspects of performance as measured functions of independent variables, but also the analytical dissection of the variation of performance. There are suggestive indications of this sort of thing in certain series of measurements of growth (*cf.* Castle, 1931; Green, 1931; Waters, 1931), but the mere variability of weight in a population is not exactly the same sort of thing as that which we here consider; there has been a tendency to confuse variability of weight with variation in rate of growth.

The form of the θ -log $\sin \alpha$ curve for the F_1 rats ($A \times B$) resembles in several respects that produced by the attachment of a small mass posteriorly on the rat's back (Crozier and Pincus, 1929-30, *b*; 1931-32, *b*, *d*). This leads to the suggestion that in the F_1 individuals there has been brought about some proportionate unbalance of development, such that it is as if a relatively inert mass were attached to the rat posteriorly. There are several ways in which this might be brought about if diverse developmental processes in the F_1 individuals can be regarded as failing to "keep in step" with entire harmony. Such a general notion could be made to account for most of the qualitative phenomena attributed to hybrid vigor or heterosis. In the present case it suffices to appeal to a relative increase in the weight of the hind quarters of the animal, or, more reasonably, to a failure of the tension receptors there involved to keep up with the increase in the mass associated with them. The distortion of the θ - α curve cannot be accounted for by any mere increase in total weight of individual, because the absolute weights of the individuals tested are no greater than with A or B parents; and because variations of weight of individuals in one pure line, within limits 14 to 25 gm., produce no such distortions,—nor do they bring about the observed alterations of the indices of variation. In ordinary development, as attested by a diversity of experiments, θ for rats of a given race is independent of weight of individual, as is also the percentage of modifiable variation of θ (*cf.* Crozier and Pincus, 1931-32, *a*; and data in subsequent papers). This must mean that during growth increase of the total mass carries with it (within the limits of observation which here concern us) its proportionate equipment of the sensory elements involved in geotropic orientation. In the growth of F_1 hybrids of A and B , however, these developmental processes may fail to keep pace. This effect is chiefly manifest, at age 13 days, in ♀♀ from $A \sigma \times B \varphi$, although the ♀♀ involved weighed about 1.0 gm. less than their ♂♂ litter-mates. The distortion in question could be produced by a very small uncompensated mass, that is, a very small mass unprovided with its proportion of receptors and located posteriorly. From the known distortion produced by masses of 2 gm. and less so located, it can be estimated that an uncompensated load of 0.5 gm. or less would be effective to the extent observed. In further generations produced by matings of F_1 with the B parents, this

small effect could easily be completely suppressed. If this supposed disharmony of development is due to hybridization, its effects should largely disappear when the backcross progeny of $F_1 \times B$ are examined, provided the essential *genetic* features of the case have to do with the dominance of B race types of quantitative distributions of receptor thresholds.

VI

The proper test of these interpretations is given by the behavior of the backcross individuals obtained in matings of the F_1 rats with A and with B parents respectively. The test is actually a severe and critical one. Ideally we should find that θ -curves from progeny of $F_1 \times B$ are practically indistinguishable from one another on the basis of sex, and indistinguishable from those of the B stock. The points of correspondence must include: threshold slope for significant response; magnitude of orientation-angle at threshold slope of surface; the shape of the curve; the index of variation (P.E. θ vs. $\sin \alpha$); variability number; total variation; and percentage of total variation which is modifiable. The offspring of $F_1 \times A$, however, are expected to exhibit a diversity of θ - α curves, as a consequence of segregation of the genes brought together in the original cross. The $F_1 \times B$ population should be homogeneous, the $F_1 \times A$ population distinctly heterogeneous, as regards the relation of θ to α ; and these facts should also be revealed by the behavior of the variability numbers and other indices of scatter of θ . Neither backcross should show so prominently the peculiarities of the distorted θ - α curve seen in F_1 . Thus a rather intricate set of conditions is imposed, which must be satisfactorily met individually and as a group, if the analysis is to stand.

We consider here only the behavior of backcross individuals obtained by matings of F_1 with B_{18} rats. The $F_1 \times A$ cross is dealt with in a succeeding paper. We may be allowed to anticipate the outcome to the extent of stating that the $F_1 \times A$ rats *are* a heterogeneous assemblage as concerns $\Delta\theta/\Delta\alpha$, threshold for orientation, and other properties. The $F_1 \times B$ individuals, which alone concern us here, provide however an inescapably convincing demonstration of their essential uniformity as a group, and of the absence in them of

TABLE VI
Orientation-angles for individuals produced in two litters of the backcross generation $F_1 \times B_1$; $n = 20$ in each case.

	α°	15°	20°	25°	35°	45°	55°	70°
I ($F_1 \varphi \times B_{18}\sigma^7$)	11 σ^7	56.4 \pm 2.19	59.3 \pm 2.08	65.0 \pm 1.50	73.7 \pm 0.805	76.7 \pm 1.11	81.7 \pm 0.980	83.4 \pm 0.678
	14"	56.1 \pm 2.33	60.4 \pm 1.98	64.2 \pm 2.12	72.7 \pm 1.61	75.9 \pm 1.32	82.1 \pm 0.960	84.7 \pm 0.591
	*17"	55.3 \pm 1.90	61.2 \pm 2.46	64.5 \pm 1.50	73.4 \pm 1.30	77.8 \pm 1.32	82.3 \pm 0.814	84.4 \pm 0.736
	*18"	55.5 \pm 2.18	60.6 \pm 1.81	63.9 \pm 1.45	72.4 \pm 1.23	78.0 \pm 0.805	82.2 \pm 0.930	84.7 \pm 0.465
	*19"	57.6 \pm 1.78	61.2 \pm 1.94	62.8 \pm 1.78	72.2 \pm 0.911	78.7 \pm 0.650	82.3 \pm 0.795	85.6 \pm 1.31
	10 φ	55.1 \pm 1.55	61.6 \pm 1.42	62.6 \pm 1.56	72.3 \pm 1.42	77.6 \pm 1.02	81.9 \pm 0.485	83.7 \pm 0.679
	12"	56.8 \pm 2.91	60.9 \pm 2.03	63.4 \pm 1.87	74.1 \pm 1.39	79.0 \pm 1.03	80.4 \pm 0.844	83.8 \pm 0.679
	13"	56.8 \pm 2.52	60.6 \pm 2.46	63.3 \pm 1.62	74.8 \pm 1.28	78.2 \pm 0.970	80.7 \pm 1.12	85.0 \pm 0.543
	15"	55.8 \pm 1.79	60.8 \pm 1.56	63.8 \pm 1.89	75.8 \pm 1.23	77.4 \pm 1.31	81.5 \pm 1.69	83.9 \pm 0.708
	16"	55.6 \pm 2.28	61.2 \pm 1.37	65.2 \pm 1.56	72.1 \pm 1.29	77.7 \pm 1.26	81.0 \pm 0.718	83.8 \pm 0.756
II ($F_1 \varphi \times B_{11}\sigma^7$)	41 φ	55.6 \pm 1.98	59.1 \pm 2.80	63.5 \pm 2.32	73.2 \pm 1.13	77.8 \pm 1.12	81.8 \pm 0.631	82.0 \pm 0.824
	42"	52.8 \pm 2.35	60.1 \pm 1.95	61.9 \pm 2.16	71.7 \pm 1.57	77.3 \pm 1.23	80.7 \pm 0.941	83.9 \pm 0.668
	43 σ^7	56.6 \pm 2.26	64.4 \pm 1.85	65.8 \pm 1.74	74.0 \pm 1.12	79.2 \pm 0.985	80.4 \pm 0.892	82.3 \pm 0.756
	44 φ	57.3 \pm 2.52	61.0 \pm 1.73	65.2 \pm 2.22	71.5 \pm 1.60	77.9 \pm 1.13	80.6 \pm 0.970	82.4 \pm 0.795
	45"	54.7 \pm 2.46	59.1 \pm 2.14	62.9 \pm 2.44	71.1 \pm 1.28	76.2 \pm 1.67	81.9 \pm 1.10	83.1 \pm 0.863

*Nursed by $A_{15}\varphi$.

phenotypic segregation of factors concerned with the determination of orientation-angle by $\sin \alpha$. In other words, the evidence of dominance of properties of the *B* race over corresponding properties of *A*, as involved in geotropic orientation, is thoroughly substantiated by the conduct of the ($F_1 \times B$) individuals. The curve θ vs. $\log \sin \alpha$ (Fig. 16, Table VII) is in fact indistinguishable from that for the *B* strain. This justifies our contention that the curious distortions of the curve for the F_1 rats (Fig. 1), aside from the disturbances of σ_θ , are due to the prevalence of conditions peculiar to the individuals of that generation, being perhaps of a purely developmental order, and are in no proper sense of a genetic character.

TABLE VII

Mean angles of orientation for progeny of matings of F_1 ($A \times B$) with *B*, two series (cf. Table VI); Series I, $N = 10$; Series II, $N = 5$; $n = 20$ throughout. Weighted mean θ 's for race *B*, at these values of α , are included.

α , degrees	θ , degrees				
	<i>B</i>	$F_1 \times B$, I	Δ	$F_1 \times B$, II	Δ
15	55.32	56.03 \pm 1.94	+0.29	54.82 \pm 0.952	+0.50
20	60.72	60.78 \pm 1.77	-0.06	60.65 \pm 0.860	+0.07
25	64.97	63.75 \pm 1.56	-1.22	63.80 \pm 0.908	+0.17
35	72.36	73.05 \pm 1.22	+0.69	72.33 \pm 0.559	+0.03
45	77.64	77.72 \pm 1.12	+0.08	77.62 \pm 0.510	+0.02
55	82.08	81.50 \pm 0.868	-0.58	80.95 \pm 0.372	+0.55
70	84.15	84.13 \pm 0.580	-0.02	82.60 \pm 0.394	+1.55

The data for rats examined in the cross ($F_1 \times B$) are summarized in Tables VI and VII, graphically in Figs. 15 and 16. In Fig. 16 it is apparent that the properties of the *B* race are completely reproduced as concerns threshold α , θ at this slope of surface, $\Delta\theta/\Delta \log \sin \alpha$ throughout, and in the absence of correlations with sex or weight.

The agreement of ($F_1 \times B$) with *B*, as regards mean θ 's, is remarkably close,—in fact (Table VII) it is closer than the P.E.'s might indicate as likely to happen; this is presumably due to the "internal averaging" brought into play in obtaining the mean figures, but is fully in accord with the consistency of performance in the various individuals concerned (Table VI). The nature of the divergencies from the values for *B* indicates that the chances of this

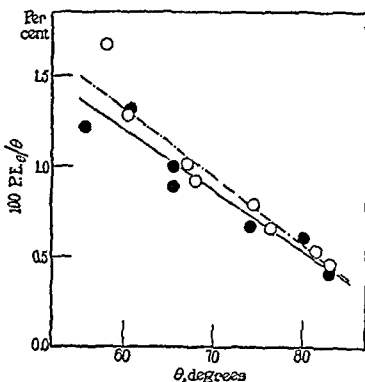


FIG. 14

FIG. 14. $100 \text{ P.E.g}/\theta$ vs. θ , F_1 ($A \sigma \times B \varphi$); open circles, Litter I; solid circles, Litter II. $V.N._g$, I = 1.54; II = 1.35.

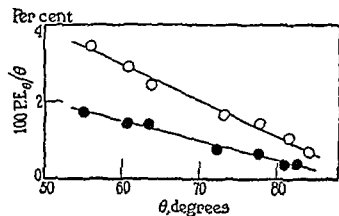


FIG. 15

FIG. 15. $100 \text{ P.E.g}/\theta$ vs. θ , ($F_1 \times B$) backcross, Litters I (open circles), II (solid circles). For I, $V.N. = 2.00$, modifiable variability = 67.6 per cent; for II, $V.N. = 2.78$, modifiable variability = 65.5 per cent.

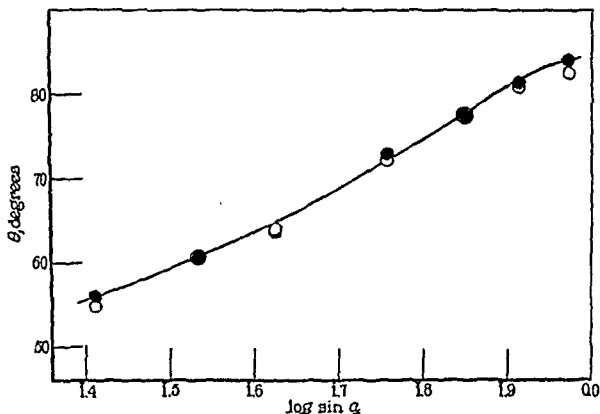


FIG. 16. θ vs. $\log \sin \alpha$ for Series I (solid circles) and II (open circles) of $F_1 \times B$ backcross individuals. The smooth curve is that for the weighted means of the B grandparents. See text. (It will be noticed that the terminal point, $\alpha = 70^\circ$, for Series II, falls a little below that called for by the curve. It will also be noticed that the probable error for this mean—cf. Table VII—is too high; this signifies that in a certain small number of cases slipping or some other "error" entered into certain of the measured trails to a barely detectable extent.)

kind of agreement occurring "by accident" are vanishingly small, particularly if it is remembered that it is the *relative* departure of mean θ which is significant. The variability indices for these individuals are:

$$\begin{array}{rcl} & (F_1 \times B) & \\ \hline \text{V.N. } \log \sin \alpha & = & 2.78 \\ \text{modifiable variation} & = & 65.5 \text{ per cent} \\ \text{total variation} & = & 1.67 \\ \text{V.N. } \theta & 2.58 & \\ \text{modifiable variation} & = & 63.4 \text{ per cent} \\ \text{V.N. } \sin \alpha & 1.11 & \end{array}$$

As with F_1 , the index $\Delta P.E._\theta / \Delta \sin \alpha$ agrees with that for B ; V.N. is a little low, but entirely within the range obtained for B litters; the total variation also agrees; the percentage modifiable variation is *lower* than with B , but definitely higher than with F_1 . All of this agrees with the expectations already formulated, including that according to which the effect of genetic influences affecting the proportion of relative variation of θ controllable by $\sin \alpha$ should be decreased by crossing to the B stock, if these influences operating to reduce the percentage modifiable variation are indeed genetic but due to factors not connected directly with the determination of the group of receptors. Moreover, the disappearance of the distortion of the θ - α curve seen in F_1 indicates that our "disharmony of developmental processes" has been effectively overcome in the backcross generation. It is noteworthy that variability of θ is *not* increased in $(F_1 \times B)$ as compared with F_1 ; the uncontrollable moiety of the variation of θ is *decreased*. If however the comparison were to be made with the lumped data for the F_1 population, this state of affairs would be totally obscured. We believe that corresponding treatment should be possible for a number of instances of "heterosis."

VII

SUMMARY

Races of *Rattus norvegicus* labelled A and B give characteristic curves relating angle of orientation θ during geotropic progression to the inclination (α) of the surface. The orientation-angles for B are

higher at every slope, and the threshold slope for orientation is lower in *B*. When these races are cross-bred, the F_1 progeny show a θ - α curve in general corresponding to that for the *B* parent, as regards both threshold slope and magnitudes of θ .

Differences between the curve for F_1 and *B* have to do (1) with a slight but significant distortion of the curve, such that from $\alpha \approx 15^\circ$ to about $\alpha = 35^\circ$ the mean curve is slightly above that for *B*, whereas above this slope of surface, θ is consistently below that for *B*; and (2) below $\alpha = 35^\circ$ the horizontal latitude of variation in the curve θ vs. $\log \sin \alpha$ is much greater than above $\alpha = 35^\circ$. The first distortion is interpreted as due to the fact that, as a manifestation of heterosis, developmental processes which upon the one hand lead to growth in bulk in the posterior region of the body, and on the other which would lead to a proportionate development of tension receptors in the legs, fail to keep pace harmoniously. In ordinary development of individuals in a pure line, harmonious relationships between these two aspects of growth are maintained. The variation of response (θ) is not altered in F_1 , if attention is paid to the total observed variation; but the unmodifiable or uncontrollable portion of the total variation of θ , not affected as a function of $\sin \alpha$, is increased. This fact is more pronounced in female offspring from the matings $A \sigma \times B \varphi$. This effect in F_1 is interpreted as due to the introduction of modifying genetic influences, which affect *variation* of orientation. The percentage of modifiable variation is reduced from 74 per cent (mean) in *B* to about 59 per cent in F_1 .

These interpretations are checked by the behavior of offspring produced in the backcross $F_1 \times B$. The disharmony of developmental processes thought to be signaled by the distortion of the form of the θ - α curve disappears completely in these backcross individuals. It can be computed that an equivalent distortion of the θ - α curve for *B* would be produced by the posterior attachment of a mass of about 0.5 gm., unaccompanied by its proportion of receptors in the legs; in backcrossing to the *B* line, this should be reduced in the average to about "0.25 gm.," which is at about the threshold for any detectable effect of a mass added posteriorly.

The variability of θ in ($F_1 \times B$) is not different from that for *B*, or for F_1 (total variation); but, as must be expected if the unmodified

variation has been increased in F_1 by the action of modifying genetic influences not directly connected with the genes determining the numbers of tension-receptors, we find that in ($F_1 \times B$) the percentage of modifiable variation is returned half-way (65 *per cent*) toward that for the B line.

The bearing of certain considerations derived from the treatment of this case is discussed with reference to the desirability that other phenomena of "hybrid vigor" and heterosis be similarly analyzed.

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THE COMBINING WEIGHT OF GELATIN AS AN ACID

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The nature of the reactions between proteins and electrolytes has occupied the attention of many investigators. The majority support the theory that proteins when pure are definite chemical compounds and that they react with electrolytes to form highly ionizable salts. There are, however, many who choose to explain all such reactions on the basis of adsorption. This question has been extensively studied by Schmidt and his coworkers, by Cohn, and by several at The Rockefeller Institute, particularly Loeb, Hitchcock, Northrop, and Simms. Good brief summaries of the work along this line are contained in recent articles by Rawlins and Schmidt,¹ Cohn,² and Stearn.³ The authors feel, therefore, that it is not necessary to discuss the present status of the problem here.

In previous publications⁴⁻⁶ one of the authors has described work in which a study was made of the nature of the reaction of gelatin in acid solutions. The combining weight of dry gelatin in hydrochloric acid was found to be 1090 gm.

The present work is a study of the reaction of gelatin in alkaline solutions. Cells of the following types were used:

$H_2/NaOH\ C/KCl\ (sat.)/NaOH\ C + \text{gelatin } x\ gm./H_2$ I

$Na, Hg/NaOH\ C/KCl\ (sat.)/NaOH\ C + \text{gelatin } x\ gm./Na, Hg$ II

* Rewritten from a thesis presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Michigan.

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³ Stearn, A. E., *J. Gen. Physiol.*, 1928, 11, 377.

⁴ Ferguson, A. L., and France, W. G., *J. Am. Chem. Soc.*, 1921, 43, 2161.

⁵ Ferguson, A. L., and Bacon, E. K., *J. Am. Chem. Soc.*, 1927, 49, 1921.

⁶ Ferguson, A. L., and Bacon, E. K., *J. Am. Chem. Soc.*, 1927, 49, 1934.

Liquid junction potential was reduced to a minimum by means of saturated KCl. From Type I cells was determined the change in hydroxide ion activity produced by definite quantities of gelatin. In a similar manner, the change in sodium ion activity was obtained from the Type II cells.

Materials and Apparatus

The NaOH used was prepared from sodium amalgam and distilled water which had been boiled *in vacuo*. To prepare the amalgam, mercury was covered with several inches of kerosene and sodium added in about 5 gm. lots. The amalgam was separated by means of a separatory funnel and carefully washed with distilled water. The reaction between amalgam and water was aided by a nickel cathode which dipped into the water. The cathode was short-circuited through an ammeter to the amalgam. Sodium hydroxide so formed did not give a test for either potassium or carbonate.

The gelatin used was a special ash-free material obtained from the Eastman Laboratory. It contained 12 per cent moisture.

The hydrogen electrodes were made from platinum foil about 1 cm. square. They were platinized in the usual manner. At least two electrodes were used in each chamber. It is reported in the literature that hydrogen electrodes cannot be used satisfactorily in gelatin solutions, and it was found in this work that after continued use there was evidence of contamination; but the difficulty was easily removed by treating the electrodes with fuming nitric acid and then using them as cathode in the electrolysis of a dilute NaOH solution. After such treatment the electrodes invariably checked to within less than 0.03 mv.

Amalgam electrodes have been described by many authors. It was found that the presence of gelatin in the solution rendered the potential of these electrodes less constant so that reliable measurements could not be taken in the more dilute NaOH solutions nor in solutions containing much gelatin.

The measuring apparatus included a Type K potentiometer and necessary accessories. The standard cell was calibrated by the Bureau of Standards. The temperature was regulated at $25^{\circ}\text{C.} \pm 0^{\circ}.01$.

Cells Used and Experimental Procedure

The cell assembly for Type I is represented in Fig. 1. Flasks of about one liter capacity were provided with five necks to accommodate hydrogen electrodes, gas inlet, and outlet, etc. Flask A contained pure NaOH solution; A' contained NaOH of the same concentration as A but in addition a known weight of gelatin.

A uniform method of procedure was adopted in making the gelatin NaOH solutions. The desired weight of gelatin was placed in a beaker and an amount of distilled water sufficient to dissolve it added. The water was kept at about 35°C. and frequently stirred. This solution was then transferred to a calibrated

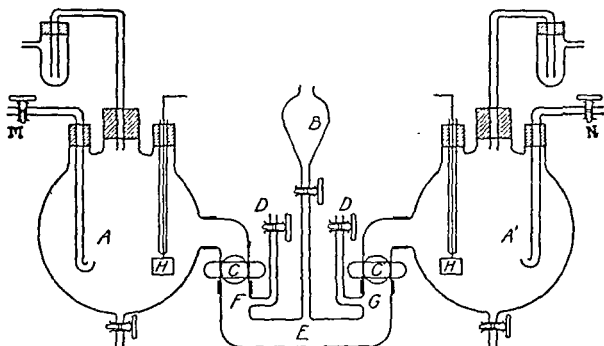


FIG. 1. The cell assembly for systems of the type
 $\text{H}_2/\text{NaOH } C//\text{NaOH } C + x \text{ gm. gelatin}/\text{H}_2$

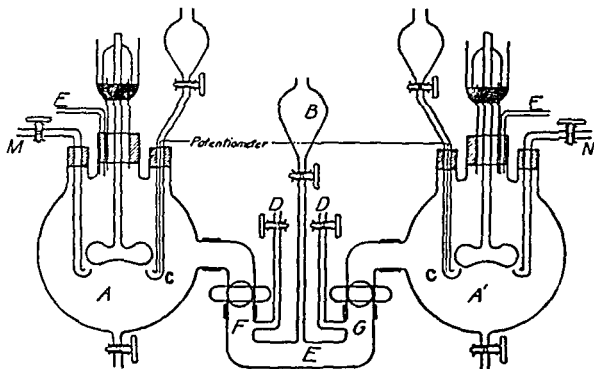


FIG. 2. The cell assembly for systems of the type
 $\text{Na}_4\text{Hg}/\text{NaOH } C//\text{NaOH } C + x \text{ gm. gelatin}/\text{Na}_4\text{Hg}$

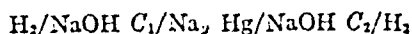
liter flask. The amount of NaOH of known concentration required to give the desired concentration was added and the whole diluted to 1 liter. The intermediate vessel was designed to reduce so far as possible the liquid junction poten-

tial. It was filled through *B* with saturated KCl to the level of the openings *F* and *G*. The junction, say at *F*, was then formed by opening *C*. Any entrapped air escaped through the flask. The flow of hydrogen was temporarily shut off and stop-cocks *C* and *C'* opened each time a potential measurement was made. Several readings were taken at intervals of about 1 hour. The cell assembly for Type II systems is represented in Fig. 2. The equipment is similar to Type I except for the additions required by the amalgam electrodes. The amalgam was allowed to drip from the openings *C* at the rate of about two drops per second. Vigorous stirring of the solution directly in contact with the amalgam was necessary. During each set of readings the potential remained constant to within 0.2 mv. The amalgam which collected in the bottom of the flasks was immediately removed. The presence of gelatin greatly increased the rate of decomposition of the amalgam so that reliable values could be obtained only in the highest concentration of NaOH used and for low concentrations of gelatin. In the high concentrations of gelatin the solutions were too viscous to permit effective stirring. In all cases recorded, however, the potentials are the averages of four sets of readings and were reproducible to a few tenths of a millivolt.

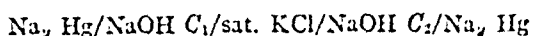
It was found necessary to protect all glass which would otherwise come in contact with NaOH solution by a coating of paraffin.

Experimental Results

The results with Type II cells are represented graphically by Curve E_{Na} of Fig. 5. In all cases the potential was about 2 mv. irrespective of the amount of gelatin. The direction of the potential was such that it can be accounted for by a decrease in sodium ions in the chamber containing gelatin; but it is possible, also, that the saturated KCl did not eliminate completely diffusion potential. To learn more about this point two cell systems were used which may be represented in the following manner:



and



If it be assumed that the saturated KCl eliminates diffusion potential completely and that the activity of the sodium ion is equal to that of the hydroxide ion in NaOH of a given concentration, then the potential of the first system should be just double that of the second. The latter assumption is probably true at the concentration used.⁷ When

⁷ Lewis, G. N., and Randall, M., *Thermodynamics*, New York, McGraw-Hill Book Co., Inc., 1923, 381.

C_1 was 0.0033 N and C_2 0.1 N, the discrepancy in values was found to be about 2.4 mv. For smaller differences in NaOH concentrations the potentials would probably be less. It may be assumed, there-

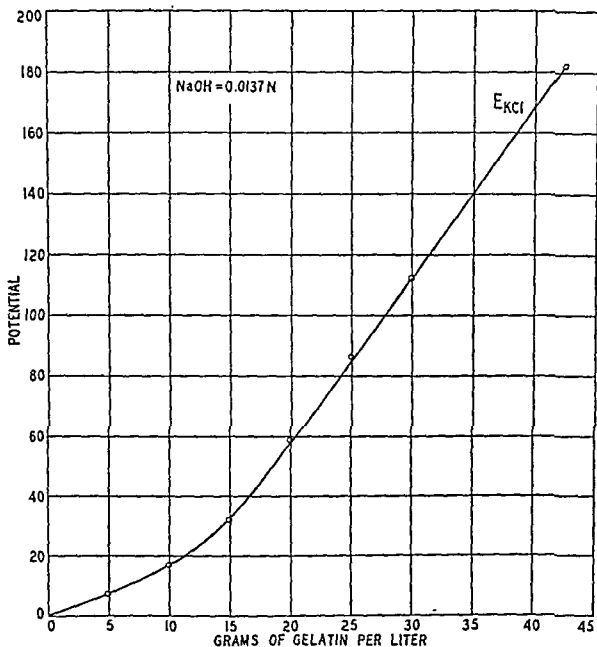


FIG. 3. Curve E_{KCl} shows the increase in potential of the cell $H_2/NaOH\ 0.0137\ N//NaOH\ 0.0137\ N + x\ gm.\ gelatin/H_2$ as x is increased.

fore, that the E_{Na} potentials for System II were due to uneliminated junction potentials. It is safe to conclude that the change in sodium ion concentration produced by the action of gelatin upon NaOH is

less than that represented by a potential of 2 mv. and is probably entirely insignificant.

Three concentrations of NaOH were used and the data are represented as curves in Figs. 3, 4, and 5. The curves must all pass through the origin, since the two halves of the cells are identical when no gelatin is present. If gelatin reacts as an acid with NaOH, then,

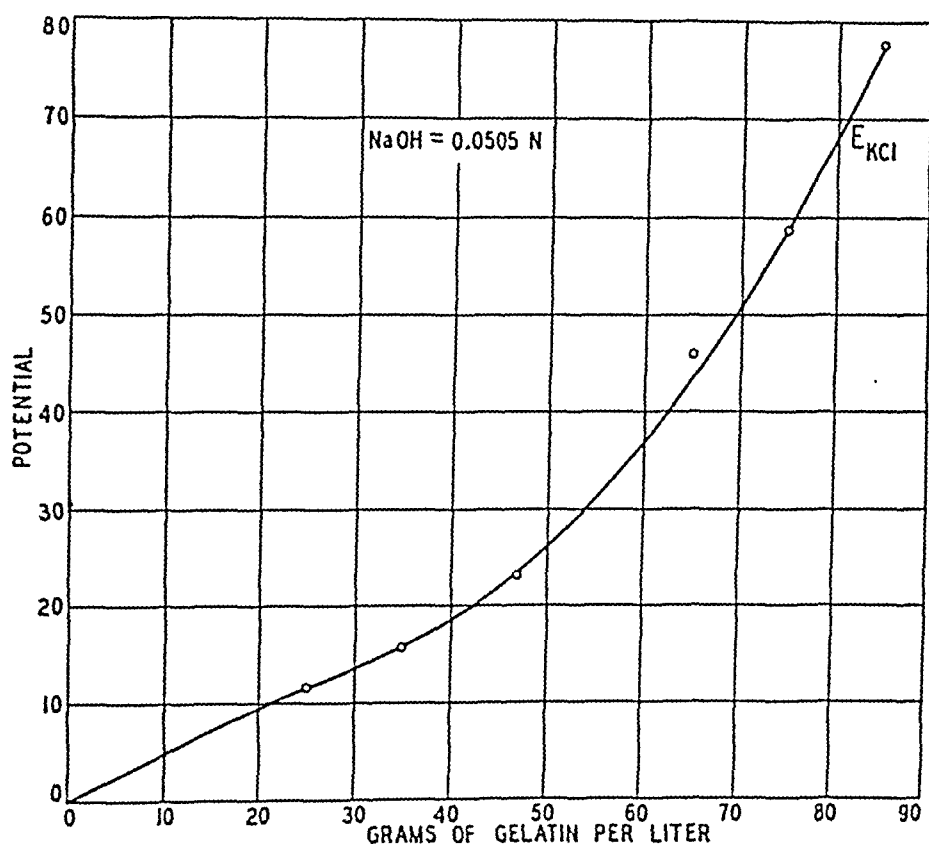


FIG. 4. Curve E_{KCl} shows the increase in potential of the cell $H_2/NaOH\ 0.0505\ N/NaOH\ 0.0505\ N + x\ gm.\ gelatin/H_2$ as x is increased.

in the chamber containing gelatin, the hydroxide and thus the hydrogen ion concentration is changed. The potentials represented by the curves are due only to changes in hydrogen ion concentration produced in this manner. The potentials should be given by the formula

$$E_{KCl} = \frac{RT}{NF} \ln \frac{a'_H}{a''_H} = \frac{RT}{NF} \ln \frac{a''_{OH}}{a'_{OH}} = \frac{RT}{NF} \ln \frac{C''_{OH} \gamma''}{C'_{OH} \gamma'}$$

where the activity, concentration, and activity coefficient of the OH ions in the pure NaOH solution are represented respectively by

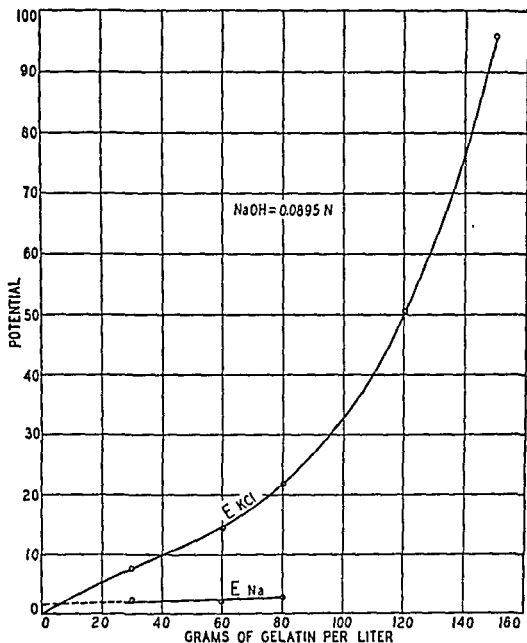
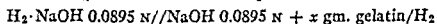
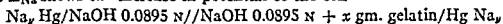


FIG. 5. Curve E_{KCl} shows the increase in potential of the cell



as x is increased.

Curve E_{Na} shows the increase in potential of the cell



as x is increased.

a_{OH}^* , C_{OH}^* , and γ^* ; and in the solution which originally had the same OH ions activity, but to which known quantities of gelatin were

added, the resulting activity, concentration, and activity coefficient of OH ions are represented by a'_{OH} , C'_{OH} , and γ' .

It was pointed out above that the potentials with sodium amalgam electrodes showed the sodium ion activity to be unchanged by the addition of gelatin. This means that the sodium gelatinate formed is highly dissociated. It is not unreasonable to assume therefore that the activity coefficient of the OH ions remains unchanged as gelatin is added to the NaOH. Cohn has shown that this is the case when NaOH is neutralized by four acids,—sulfuric, oxalic, glutamic and aspartic. If this is true then $\gamma' = \gamma''$ and the actual concentration of NaOH remaining unneutralized after each addition of gelatin is given directly from the potential measurements and the above formula.

This is practically the same method as introduced by Bugarsky and Liebermann⁸ and later used by Robertson.⁹ Another method introduced by Tague¹⁰ and adopted by several involves the measurement of a "blank," *i.e.*, the amount of reagent required to change the water to the same pH as the solution under consideration. As Tague pointed out, it is not necessary to take into account the degree of ionization by this method. It must be remembered, however, that the hydrogen electrode measures activity, and in the "blank" employed there is nothing present but NaOH, while in the neutralized system there is ionized salt in addition. In calculation, therefore, it must be assumed that the ionized salt has no influence upon the activity of the hydroxide ions. This source of error has been pointed out by Cohn and Berggren.¹¹

In the determination of base combining power of proteins, Cohn and Berggren measured the potential of the hydrogen electrode in the solution containing base and protein against a 0.1 N calomel electrode. They also measured or calculated the pH of the pure NaOH solutions. The pH was calculated by the equation

$$\text{pH} = \frac{\text{E.M.F. observed} - \text{E.M.F. 0.1 N calomel}}{0.001933 T}$$

⁸ Liebermann, L., *Arch. ges. Physiol.*, 1893, 72, 51.

⁹ Robertson, T. B., *The physical chemistry of the proteins*, New York, London, Bombay, Calcutta, and Madras, Longmans Green and Co., 1918.

¹⁰ Tague, E. L., *J. Am. Chem. Soc.*, 1920, 42, 173.

¹¹ Cohn, E. J., and Berggren, R. E. L., *J. Gen. Physiol.*, 1924-25, 7, 45.

From this they obtained p_{OH} from the relation

$$pH + p_{OH} = pK_W$$

and ultimately the stoichiometric concentration by the equation

$$(NaOH) = \frac{(OH)}{\gamma}$$

The activity coefficient is thus involved in all of their calculations of bound NaOH. This would not have been necessary, however, if they actually measured the pH of each of the pure NaOH solutions used and had assumed that the activity coefficient of the hydroxide ions remained constant. In some cases they made such measurements and attempted to show that different values for the base binding power of casein are obtained according to whether Robertson's or their method of calculation is used. If no errors are involved in the potential measurements due to a lack of elimination of boundary potential in the one case and the assumption that the boundary potential between the calomel electrode and each of the other solutions is zero or the same for both in the other case, then the two methods must give the same value since the activity coefficient does not enter into the calculation in either case and the same formula is used.

The combining weights obtained for various quantities of gelatin are recorded in Table I. The first value for each concentration is not included in the averages, since the per cent error in the measurements for these values is much greater than for the others.

Combining weights were not calculated for the larger amounts of gelatin, because these are greater than that required for complete combination. The quantities of gelatin for complete combination are 21.3 gm. for the 0.0137 *N* NaOH, 78.6 gm. for the 0.0503 *N*, and 139 gm. for the 0.0895 *N*.

It should be observed: first, that the combining weight of gelatin obtained here is independent of the concentration of NaOH; second, that in each of the three concentrations of NaOH the combining weight of gelatin is independent of the ratio of base to gelatin. In other words, the combining weight of gelatin remains practically constant through a wide pH range. These facts cannot be accounted for on the basis of adsorption.

For quantities of gelatin near the end-point the amount of NaOH that remains is too small to prevent hydrolysis and the method does not apply. In fact the last combining weight value recorded for 0.0137 N is higher than all others obtained and this is due probably to hydrolysis.

Various values for the combining weight of gelatin as an acid are recorded in the literature. The values appear to depend upon both the method used and the investigator.

In his extensive and highly valuable review of the physical chemistry of the proteins, Cohn¹² states, "A recalculation of Loeb's¹³ and Hitch-

TABLE I
Combining Weights of Gelatin (not Corrected for Moisture)

Conc. of NaOH 0.0137 N		Conc. of NaOH 0.0505 N		Conc. of NaOH 0.0895 N	
Gelatin per liter	Combining weight	Gelatin per liter	Combining weight	Gelatin per liter	Combining weight
<i>gm.</i>		<i>gm.</i>		<i>gm.</i>	
5	1430*	25	1360*	30	1310*
10	1475	35	1530	60	1570
15	1520	47	1560	80	1570
20	1620	65	1555	120	1570
25		75		150	
30		85			
42.5					
Average . . .	1538		1548		1570

* These values were not included in averages.

cock's¹⁴ electromotive force measurements yield 56, 57, and 56×10^{-5} mols per gram as the combining capacity. Greenberg and Schmidt¹⁵ report an almost identical value of 60×10^{-5} mols per gram, and this is the highest base combining capacity that Atkin and Douglas¹⁶ measurements reveal upon recalculation."

¹² Cohn, E. J., *Physiol. Rev.*, 1925, 5, 349.

¹³ Loeb, J., *J. Gen. Physiol.*, 1920, 3, 85.

¹⁴ Hitchcock, D. I., *J. Gen. Physiol.*, 1923, 6, 457.

¹⁵ Greenberg, D. M., and Schmidt, C. L. A., *Proc. Soc. Exp. Biol. and Med.*, 1924, 21, 281.

¹⁶ Atkin, W. R., and Douglas, G. W., *J. Soc. Leather Trades' Chemists*, 1924, 3, 584.

Loeb, in the article referred to, makes no attempt to calculate the combining weight; in fact the data given would have to be corrected for the amount of base required to give the water alone the respective pH values. The same statements apply also to the data of Hitchcock to which reference is made.

Concerning the data of Atkin and Douglas, Miss Lloyd in her book, *Chemistry of the proteins*, published in 1926, makes the statement that their data show "combination of gelatin and bases takes place in two stages, the first in which 1 gm. combines with about 30×10^{-5} equivalents of base, the second in which 1 gm. combines with 80×10^{-5} equivalents." In another place, however, Miss Lloyd states, "Atkin and Douglas find 30×10^{-5} for a first stage of titration and 70×10^{-5} for a second stage." This discrepancy in statements is easily understandable since the pH combination curve shows no sharp break and it is largely a matter of choice whether to take 70×10^{-5} which is the value for pH 11 or 80×10^{-5} which is the value for pH about 13. I do not, however, understand how Cohn gets the low value 60×10^{-5} from their data as "The highest base combining capacity that Atkin and Douglas' measurements reveal upon recalculation."

In the article by Greenberg and Schmidt, referred to by Cohn, they estimate the base combining capacity of gelatin upon the recent analysis of gelatin by Dakin.¹⁷ He reports the base binding power of the aspartic acid present in gelatin to be 26×10^{-5} and of glutamic acid to be 39×10^{-5} or a total of 65×10^{-5} . From this he subtracts 23×10^{-5} , the amide nitrogen, thus leaving a combining capacity of 42×10^{-5} . They report that the actual found binding capacity at pH 11 is 60×10^{-5} . Greenberg and Schmidt do not include their data but state, "Our method of estimating the base combining power of the proteins was carried out according to the procedure which has previously been used by Tague¹⁸ for amino acids and by Loeb and Hitchcock for proteins. On account of the logarithmic increase in pH on addition of alkali the method is not capable of a very high degree of accuracy at high alkalinity."

In view of this analysis of the literature one might question the

¹⁷ Dakin, H. D., *J. Biol. Chem.*, 1920, 44, 499.

¹⁸ Tague, E. L., *J. Am. Chem. Soc.*, 1920, 42, 173.

statement of Cohn, "The maximum base combining capacity of gelatin is extremely well known." Later developments render the statement even more questionable and also the combining value about 56×10^{-5} which he appears to accept. Simms¹⁹ has carried out what appears to be a highly accurate electrometric titration of gelatin throughout the range from about pH 1.5 to pH 11.5. He indicates a maximum base combining capacity of 70×10^{-5} .

The conductometric method has been applied in what appears to be a carefully performed series of experiments by Stearn.²⁰ Base is titrated with gelatin and also gelatin with base. By the former

TABLE II
Summary of Values for Equivalent Weight of Gelatin As an Acid

Author	Date	Combining weight
Loeb.....	1920	1790 recalculated by Cohn
Hitchcock.....	1923	1790 " " "
Greenberg and Schmidt.....	1924	1670
Atkin and Douglas.....	1924	1670 according to Cohn
" " ".....	1924	3330 (pH ₂) and 1250 (pH ₁₁) according to Lloyd or 1430 (pH ₁₃)
Estimated from acid content as determined by Dakin.....		1540 or 2380
Simms.....	1928	1430
Stearn.....	1928	1320
".....	1928	1360
Rawlins and Schmidt.....	1929	1430
Ferguson, Schluchter.....	1931	1370

method he obtains for the combining capacity 75.8×10^{-5} and by the latter 73.5×10^{-5} .

Schmidt and his coworkers have studied the combining power of gelatin for acid and basic dyes. They find that the maximum combining power is found only in strongly acid or basic solutions. Rawlins and Schmidt²¹ show that the combining power of gelatin for methylene blue, safranin γ , and induline scarlet is approximately the same. They give for pH 11 a combining capacity 70×10^{-5} .

¹⁹ Simms, H. S., *J. Gen. Physiol.*, 1927-28, 11, 629.

²⁰ Stearn, A. E., *J. Gen. Physiol.*, 1928, 11, 377.

²¹ Rawlins, L. M. C., and Schmidt, C. L. A., *J. Biol. Chem.*, 1929, 82, 709.

An uncertainty that enters into many of the values given is the condition of the gelatin. It is seldom stated whether dry gelatin was used. Gelatin ordinarily contains about 10 per cent or more moisture. Stearn states specifically that his values are for dry gelatin.

The average of all the values for the combining weight obtained in the present investigation, corrected to 1 gm. of dry gelatin, is 73×10^{-5} . The gelatin used contained 12 per cent moisture.

Since this work was prepared for publication the excellent recent work of Hitchcock²² has come to the attention of the authors. The combining capacity of gelatin for NaOH is extremely indefinite according to his data, though the value 84×10^{-5} obtained with Eastman Purified Gelatin, Lot 51, is of the same order of magnitude at least as the value obtained in this work.

SUMMARY

1. The nature of the reaction between gelatin and sodium hydroxide has been studied at three concentrations of gelatin.

2. Gelatin appears to react stoichiometrically with sodium hydroxide.

3. The addition of gelatin to sodium hydroxide does not change the concentration of sodium ions in the solution.

4. A given amount of gelatin reacts with the same amount of sodium hydroxide no matter what the concentration of sodium hydroxide or ratio of sodium hydroxide to gelatin, provided there is sufficient excess of sodium hydroxide to prevent hydrolysis.

5. The combining weight of dry gelatin as an acid is found to be 1370 gm.

²² Hitchcock, D. I., *J. Gen. Physiol.*, 1931, 15, 125.

THE MOBILITY OF THE GELATINATE ION

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(From the Chemical Laboratory of the University of Michigan, Ann Arbor)

(Accepted for publication, January 5, 1932)

Many attempts have been made to determine the mobilities of large ions or charged particles of colloidal dimensions. Several methods have been used based upon the movement of charged particles under a difference of potential. They may be grouped into three classes: (1) conductivity; (2) transport numbers, (a) Hittorf method, (b) moving boundary method, (c) electromotive force method; (3) cataphoresis.

From a review of the literature on this subject one is impressed by the lack of uniformity in results obtained with different methods and by different investigators.

According to the earlier generally accepted ideas concerning ion velocities it is to be expected that protein ions should have low mobilities, and some investigators have reported low values. Hardy¹ found, by the conductivity method at 18°C., about 7.5 for the mobility of the globulinate ion. The moving boundary method gave him about 9 for the globulin ion in chloride solution and about 7 for the globulinate ion in sodium globulinate.

Pauli,^{2,3} McBain,⁴ Greenberg and Schmidt,⁵ Svedberg⁶ and their coworkers all found that the mobilities of protein ions depend upon the relative amounts of the protein and acid or base in the solution. Pauli found by the conductivity method that the mobility of the protein ion increased from about 10 in a strongly alkaline solution to a constant

* Rewritten from a thesis presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Michigan.

¹ Hardy, W. E., *J. Physiol.*, 1905, 33, 251.

² Pauli, W., *Anz. Akad. wissenschaft. Wien*, 1913, No. 24.

³ Pauli, W., *Biochem. Z.*, 1919, 99, 219.

⁴ McBain, J. W., and Salmon, C. S., *J. Am. Chem. Soc.*, 1923, 42, 426.

⁵ Greenberg, D. M., and Schmidt, C. L. A., *J. Gen. Physiol.*, 1924-25, 7, 287, 303.

⁶ Svedberg, T., *J. Am. Chem. Soc.*, 1924, 46, 2700.

value of about 30 as the protein content was increased. The boundary potential method gave him, for a solution containing 1 per cent albumin in 0.002 N HCl, a mobility of 5-8.

Scott and Svedberg, by a special arrangement of the moving boundary method, were able to measure directly the velocity of protein ions. They reported a maximum mobility of about 20 in acid solution.

By the conductivity method, Greenberg and Schmidt found for the caseinate ion at 25°C. values from 25.9 to 36.2 depending upon the alkali used. They were unable to obtain results with the moving boundary. The Hittorf method gave them values from 43 to 46.5 for the caseinate ion.

The explanations for the phenomena observed fall into two general classes. According to some the colloidal particles receive their charges through hydrolysis and adsorption, while others believe they are ions which result from the dissociation of chemical compounds. For more complete discussions of this question the reader is referred particularly to the articles by Cohn,⁷ McBain and Salmon,⁴ Greenberg and Schmidt,⁵ Pauli,⁸ Robertson,⁹ and Belden.¹⁰

For the present work the authors attempted to accumulate some information concerning the migration velocity of the gelatinate ion in various relative concentrations of sodium hydroxide. It is universally accepted that a potential difference exists at the surface of contact between two solutions of electrolytes that differ in any manner whatever. Various formulas have been developed to express the magnitude of such potentials; the complexity of the formulas increases with the complexity of the system. All such formulas are based upon the assumption that the magnitude of the potential depends upon the mobilities and concentrations of the ions in the two solutions. In systems where the boundary potential can be experimentally measured and the concentrations of all the ions and the mobilities of all but one ion are known, it is possible to calculate the mobility of that one.

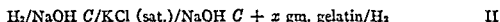
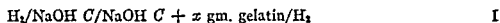
⁷ Cohn, E. J., *Physiol. Rev.*, 1925, 5, 349.

⁸ Pauli, W., *Colloid chemistry of the proteins*, Philadelphia, P. Blakistons' Sons and Co., 1922.

⁹ Robertson, T. B., In Alexander, J., *Colloid chemistry*, New York, The Chemical Catalog Co., Inc., 1928, 2, 255.

¹⁰ Belden, B. C., *J. Phys. Chem.*, 1931, 35, 2164.

The two types of cells used may be represented in this manner



The same concentration of NaOH was used on the two sides of the boundary but on one side there was in addition a known quantity (x) of gelatin. The two types of cells were identical except that in II the boundary potential was eliminated by saturated KCl solution. It is evident that the difference ($E_{\text{KCl}} - E_{\text{H}}$) between the potentials of these two cells gives the boundary potential E_B .

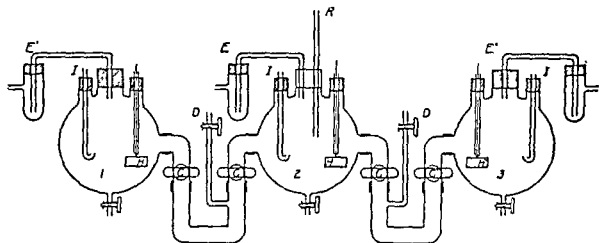
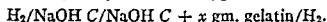


FIG. 1. The cell assembly for systems of the type



The materials used and measuring apparatus have been described in a previous paper.¹¹

The cell assembly for Type II system was the same as in Fig. 1 in the previous paper. For Type I systems the arrangement represented in Fig. 1 of this article was used. The flasks were similar to those used for Type II systems; their capacity was about 1 liter and they had five necks to accommodate the hydrogen electrodes, gas inlet, and outlet, etc. Flask 2 contained the pure NaOH solution, while Flask 1 contained NaOH of the same concentration as 1 but in addition a definite amount of gelatin. Flask 3 also contained the same concentration of NaOH but a different amount of gelatin than 1.

¹¹ Ferguson, A. L., and Schluchter, A. W., *J. Gen. Physiol.*, 1931-32, 15, 463.

This arrangement made it possible to carry on two sets of measurements at the same time. The liquid junction vessel permitted the formation of a definite horizontal boundary between the two solutions. The boundary could be renewed as frequently as desired.

To form a junction, for instance between 1 and 2, stop-cocks C' and D were opened and solution drawn out of Flask 1; C' was then closed and C opened and solution drawn out of Flask 2. The solutions in 1 and 2 were adjusted to the same level and D closed. Before taking each set of readings the hydrogen inlets were closed and stop-cocks C and C' opened. Readings were taken, (1) for boundaries that had been prepared for several minutes, (2) for freshly prepared boundaries, and (3) for flowing boundaries. The following junction was made by allowing the hydrogen to bubble slowly with C and C' open and D partly open. Potentials checked to about 0.3 mv. at the lower concentrations of gelatin, but when gelatin was present in large excess the variation reached several millivolts. Duplicate measurements were made in all cases by refilling the flasks with new solutions.

The data are represented as curves in Figs. 2 and 3. For the curves in Fig. 2 the NaOH was 0.0137 N and in Fig. 3, 0.0505 N . The data for Type I cells are represented in each figure by curves E_H , and for Type II cells by curves E_{KCl} . The E_H curves show the change in liquid junction potential with increasing amounts of gelatin, and are obtained by plotting the differences between corresponding values for E_H and E_{KCl} against grams of gelatin.

The observed data can be explained on the basis of chemical reaction between gelatin and NaOH. All curves must of course pass through the origin, since, at the start the solutions are the same in all flasks. If gelatin acts as an acid and combines with the sodium hydroxide, then the rapidly moving hydroxide ions on one side of the boundary would be replaced by heavy slow moving gelatinate ions. In a previous paper¹¹ it was shown that the activity of the sodium ion is not changed by the addition of gelatin which means that the sodium gelatinate is practically completely dissociated. The replacement of hydroxide ions by gelatinate ions is, therefore, the only change that results from the addition of gelatin and the only cause for the development of a liquid junction potential. According to this view the boundary potential should increase in value until the NaOH has been entirely

neutralized by gelatin, after which further additions of gelatin should have very little or no effect. This was found to be the situation with

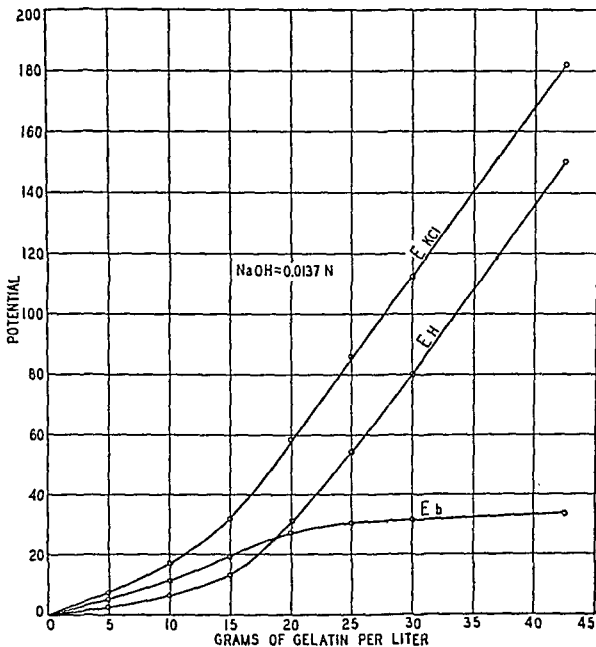


FIG. 2. Curve E_{KCl} shows the increase in potential of the cell

$H_2/NaOH\ 0.0137\ N/KCl\ (sat.)/NaOH\ 0.0137\ N + x\ gm.\ gelatin/H_2$ as x is increased. E_H is a similar curve for the cell

$H_2/NaOH\ 0.0137\ N/NaOH\ 0.0137\ N + x\ gm.\ gelatin/H_2$.

Curve E_b shows the corresponding changes in boundary potential.

0.0137 N NaOH but was not strictly the case for the higher concentration. In the 0.0505 N NaOH the boundary potential reached a maxi-

mum and then dropped for the largest amount of gelatin used. This last quantity of gelatin is more than enough for complete combinations of all the NaOH, according to the combining weight reported in a previous paper. This excess gelatin may possibly explain the decrease in potential for the last point. Some recent unpublished results show

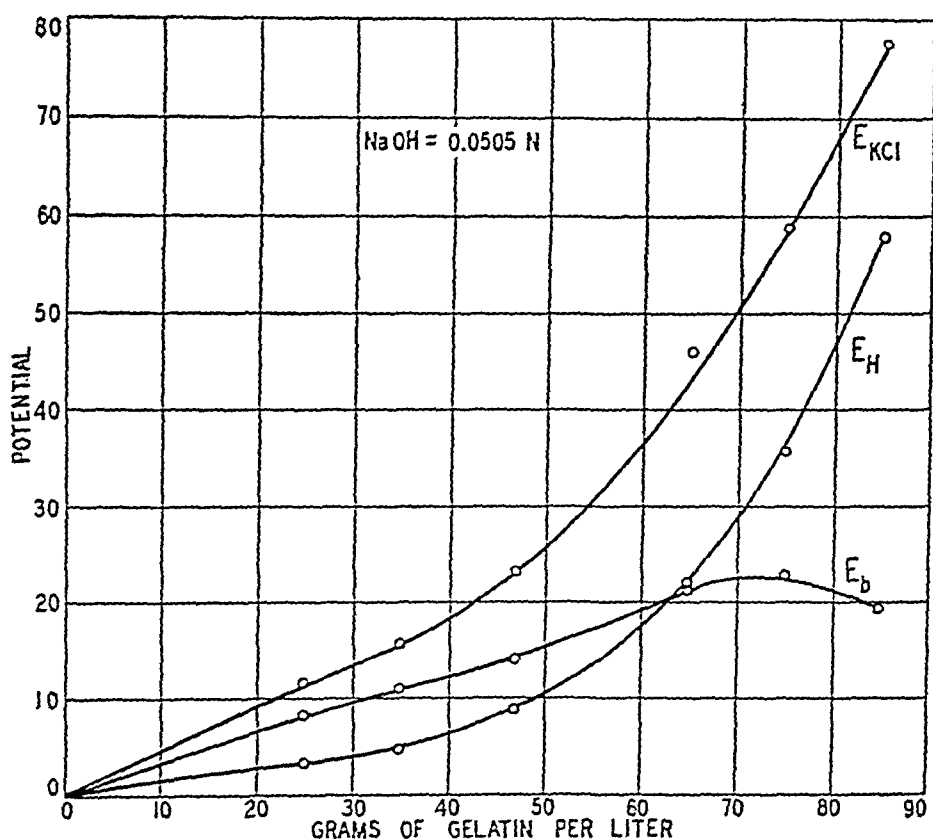


FIG. 3. Curve E_{KCl} shows the increase in potential of the cell

$H_2/NaOH$ 0.0505 N/ KCl (sat.) $NaOH$ 0.0505 N + x gm. gelatin/ H_2 as x is increased. E_H is a similar curve for the cell

$H_2/NaOH$ 0.0505 N/ $NaOH$ 0.0505 N + x gm. gelatin/ H_2 .

Curve E_b shows the corresponding changes in boundary potential.

definitely that an excess of a very weak base in a system similar in fundamental respects to the one used in this work caused a decrease in boundary potential when the solution on the other side of the boundary was an acid. An explanation of this phenomenon will be given in a later paper.

If the assumption is correct that gelatin reacts chemically with the sodium hydroxide to form a highly dissociated salt, then the concentrations of all the ions in both solutions may be calculated from the

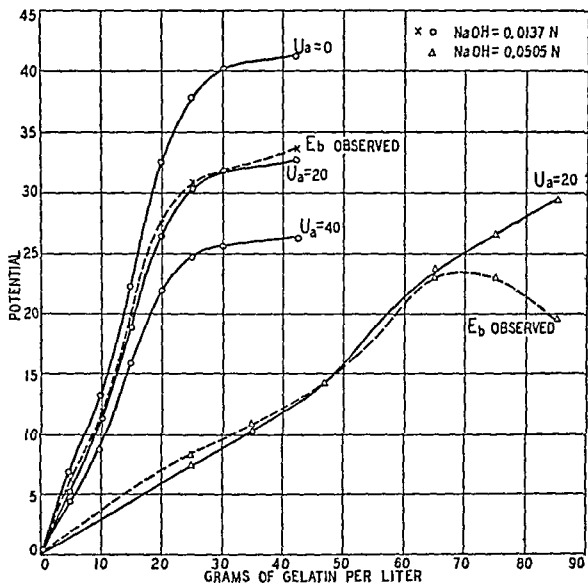


FIG. 4. Curves which represent calculated and measured values for boundary potential. $U_a = 0$, $U_a = 20$, and $U_a = 40$ represent values calculated by means of the Henderson equation and based upon the assumption of 0, 20, and 40 as the mobility of the gelatinate ion. The E_b (observed) represents the measured values. The curves with circles apply to 0.0137 N NaOH and with triangles to 0.0505 N NaOH.

data obtained. The mobilities of all the ions are known except the gelatinate ion. In all cases the cations are the same on both sides of the boundary and have the same concentrations, as shown in previous

work. The total anion concentrations in the two solutions is the same. For this special type of boundary the Henderson formula reduces to the form.

$$E_b = \frac{RT}{F} \ln \frac{U + V_1}{U + V_2}$$

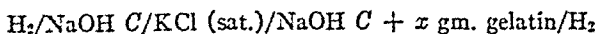
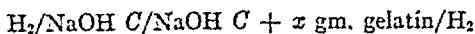
In order to calculate E_b by this formula, however, it is necessary to assume some value for the mobility of the gelatinate ion. Calculations of the potential of each boundary experimentally determined for the 0.013 N NaOH solutions were carried out with various assumed mobilities, for the gelatinate ion. The calculated values for the three assumed mobilities 0, 20, and 40 as well as the measured are represented as curves in Fig. 4.

The agreement between the observed results and those calculated on the assumption that the mobility of the gelatinate ion is 20 is striking. The differences are within the limits of experimental error. The agreement is somewhat less satisfactory for 0.0505 N NaOH solutions though it is about within the limits of experimental error except for the last two points, and an explanation has been suggested above for the low observed values.

It appears from this work that the mobility of the gelatinate ion does not depend upon the relative amounts of base and gelatin in the solution or the concentration of NaOH within the limits used. As pointed out above, others have reported a marked change in mobility with a change in relative amounts of base and gelatin. The authors suggest that possibly the difference may be due to the length of time the solutions were prepared before measurements were taken. Rawlins and Schmidt¹² have shown that days may be required to reach equilibrium in gelatin solutions. For this work the solutions were made up at 35°C., which greatly hastens equilibrium, and allowed to stand several hours and in most cases days before they were used.

SUMMARY

1. Many measurements were made with the two systems



¹² Rawlins, L. M. C., and Schmidt, C. L. A., *J. Biol. Chem.*, 1930, 88, 271.

in which α was varied from zero to more than enough for complete combination.

2. From these measurements the change in boundary potential with quantity of gelatin present was determined.

3. The boundary potentials were calculated by means of a modified form of the Henderson equation.

4. The results indicate that the mobility of the gelatinate ion is about 20.

ON "REVERSAL" OF PHOTOTROPISM IN PHYCOMYCES

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(Accepted for publication, February 12, 1932)

I

The light-sensitive sporangiophores of the fungus *Phycomyces* respond to sudden illumination by an acceleration of growth. If the illumination is from one side only, unequal growth on opposite sides of the sporangiophore typically causes bending toward the source of light—a "positive" phototropic reaction. Such bending has been generally explained as due to greater photic action on the side more remote from the light, due to refraction and total reflection within the clear, cylindrical sporangiophore (Blaauw, 1914; Oehlkers, 1926). Senn's (1908) careful determination of the light paths within structurally comparable plant cells, such as *Vaucheria*, supports this view.

Reversal of the usual positive phototropism of *Phycomyces* has been described as occurring in two cases: (1) Buder (1920) immersed the sporangiophores in oil of high refractive index, and obtained "negative" bending (away from the source of light) following one-sided illumination. This experiment has been repeated with confirmation of Buder's findings. It is surprising that growth and bending of the sporangiophore continue for over an hour in spite of the unfavorable circumstances. (2) Oltmanns (1897) and Blaauw (1909) obtained negative bendings with long exposures to high intensities of light from one source. Both investigators found positive phototropism at low intensities, phototropic "indifference" or absence of bending at intermediate intensities, and at high intensities reversal of the usual phototropism. Both Oltmanns and Blaauw regarded these results as significant for the theory of phototropism: the former spoke of "optimum" intensities of light for the several phototropic reactions; the latter of a constant quantity of light (intensity \times time) necessary to evoke each type of reaction. Blaauw further described phototropic

"indifference" as due to a balance between opposed positive and negative phototropic tendencies, and compared negative phototropism to the overexposure of a photographic plate.

II

It has been shown (Castle, 1931) that phototropic "indifference" in *Phycomyces* to short exposures of light from one side is really due to equal photic action of the same kind on opposite sides of the sporangio-phore. Since no trace of negative phototropism was encountered in these experiments, the work of Oltmanns and of Blaauw was repeated.

Using a small arc-lamp (approximately 8 amp., 110 volts A.C.), a water screen 3.3 cm. thick, and a range of intensities comparable to those used by Oltmanns, negative bending was never obtained with actively-growing sporangiophores of *Phycomyces blakesleanus* ("+" strain) even after 2 hours exposure. At the highest intensities used, enough heat is transmitted by such a water screen to stop the growth of the sporangiophores within one-half hour. Using a CuSO_4 solution screen (equivalent to a layer 1 cm. thick of 6 per cent aqueous $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$; cf. Nuernbergk and Du Buy, 1930) only phototropic "indifference" was found, and the heat effect was abolished. In no case did negative bending occur.

Negative bending is obtained, however, on exposure to sufficiently intense and prolonged infra-red radiation from one side, as the radiation from an incandescent tungsten filament transmitted by Wratten infra-red filter No. 88. Negative bending is also produced by infra-red radiation from an ordinary 110 volt glow-heater run in series with a resistance at such a low temperature that no visible light is emitted. In both of these cases, bending is gradual, first becoming evident after several hours of exposure, as would be expected in a heating effect.

III

The previously reported (Oltmanns, 1897; Blaauw, 1909) reversal of phototropism of *Phycomyces* at high intensities of light was evidently due to the action of infra-red or heat radiation not adequately screened out. The light-sensitive system proper of *Phycomyces* is insensitive to wave-lengths greater than about $580 \text{ m}\mu$ (Castle, 1930-31). Oltmanns

used a water screen 6 cm. thick in conjunction with a powerful arc-lamp, while Blaauw apparently used no kind of heat screen. The experiments described here show that heat radiation alone can produce just the kind of gradual growth away from the radiant source which these workers found. Such bending was in reality negative *thermotropism*, not compounded with phototropism because the sporangiophores were symmetrically saturated with light, or phototropically "indifferent."

Reversal of the ordinary positive phototropic movement of the sporangiophores of *Phycomyces* with high intensity of illumination is therefore not a genuine phenomenon, and need not be accounted for in terms of the underlying light-sensitive system.

SUMMARY

Alleged reversal of the phototropism of the sporangiophores of *Phycomyces* by high intensities of light does not occur if infra-red radiation is properly excluded. Phototropic "indifference" alone occurs at high intensities due to equal photic action on both sides of the sporangiophore. If heat radiation is not screened out, a gradual, negative thermotropic bending takes place.

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SIMILARITY OF THE KINETICS OF INVERTASE ACTION IN VIVO AND IN VITRO

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(Accepted for publication, February 24, 1932)

Attempts to measure physiological reactions using a purely chemical method of determining the course of the reaction have produced unsatisfactory results, particularly when a quantitative identity of the reaction course of the substance in the live organism and in the extract of the non-living material has been sought. Owing to the complexity of the environment under which chemical reactions in protoplasmic systems take place, a more or less distinct line of demarcation has arisen between the realms of physiology and chemistry. Since the kinetics of the enzymic hydrolysis of sucrose has been extensively studied by workers in this laboratory and others using, however, invertase solutions, it was considered interesting to attempt a comparative study of this reaction using live yeast cells as a source of the inverting activity.

The idea of the possible similarity of invertase activity *in vivo* and *in vitro* is to be found as early as 1911 in work of Euler and Kullberg (1), who made a comparative study of the reaction under the two named conditions. Similar although not identical results in the two cases were obtained, due to inconstancy of experimental procedure and to the use of the monomolecular law as a measure of invertase activity. Since then, however, it is quite generally agreed that the hydrolysis of sucrose by invertase prepared from yeast, *Saccharomyces cerevisiae*, is not a first order reaction (2).

The following empirical equation of Nelson and Hitchcock (3) has been found to represent closely the course of this reaction for nearly all of the invertase preparations made in this laboratory.

$$N = \frac{1}{t} \left[\log \frac{100 - p}{p} + 0.002642 p - 0.0, 886 p^2 - 0.0, 1034 p^3 \right]$$

p = per cent sucrose hydrolyzed in t minutes. This equation is applicable to 10 per cent sucrose solutions at pH 3.5 to 7.0 at a constant temperature between 20° and 35°C. (4). The constancy of the value of the term N with varying values of p and t therefore serves as a criterion of a definite course of the hydrolytic reaction and any increasing or decreasing trend of the magnitude of N during a hydrolysis is indicative of a different course of the reaction. If constant values of the term N are obtained when live yeast cells are used as the hydrolytic agent the course of the reaction will thereby be shown to be identical with that resulting from the use of invertase solutions obtained from the same yeast. Likewise an identity of results of a reaction run *in vitro* and *in vivo* will show that in the case of the use of the live cells, as in the use of invertase solutions, we are measuring one reaction only in a protoplasmic system; namely, the inversion of sucrose.

EXPERIMENTAL

Hydrolysis of Sucrose by Invertase Solutions.—The invertase preparations used were obtained by autolyzing samples of the commercial brand of Fleischmann's yeast, with toluene and water. The invertase was precipitated from the autolysate with 50 per cent ethyl alcohol and extracted from this precipitate with water containing 0.01 M acetate buffer (pH 4.5).

In running the hydrolyses, 50 cc. of the enzyme were pipetted into 200 cc. of 12.5 per cent sucrose solution, containing 0.01 M acetate buffer (pH 4.5). The hydrolysis mixture was maintained at a temperature of $25^{\circ} \pm .01^{\circ}\text{C}$. 25 samples were pipetted out at various time intervals and inversion stopped by the addition of one drop of sodium hydroxide solution of such strength as to bring the pH of the sample to 8–9. After allowing time for mutarotation to occur, the samples were polarized at 25°C. in a 2 dm. tube using a mercury arc-light of wavelength 546.1 μ .

The values of N in the equation of Nelson and Hitchcock were calculated for these hydrolyses and the results presented in Table I show the constancy of this term during the course of a hydrolysis.

Hydrolysis of Sucrose by Live Yeast Cells.—In order to run quantitative hydrolyses with live yeast cells some modification of the experimental procedure was necessary. A weighed quantity of washed, pressed yeast was suspended in 100 cc. of water containing 0.01 M acetate buffer (pH 4.5). 50 cc. of this suspension were pipetted into 200 cc. of 12.5 per cent sucrose solution containing 0.01 M acetate buffer at the same pH. In order to secure a uniform suspension of yeast the

hydrolysis mixture was shaken continuously in a thermostat at $25^{\circ} \pm 0.01^{\circ}\text{C}$. Samples were removed and inversion stopped as described above and were immediately filtered by suction through porous-bottom Gooch crucibles, by which means a cell-free filtrate was obtained. The filtrates were polarized as in the preceding method.

TABLE I
Hydrolyses Using Invertase Solutions from Autolyzed Yeast
(Several duplicate hydrolyses run by Fassnacht (5))

Time	Rotation	Δ Rotation	Per cent hydrolyzed	$N \times 10^3$
<i>min.</i>	<i>degrees</i>			
0	15.66			
50	10.42	5.24	25.91	382
80	7.58	8.08	39.96	383
100	5.91	9.75	48.22	381
120	4.37	11.29	55.84	381
140	2.99	12.67	62.66	381
170	1.27	14.39	71.17	380
205	-0.33	15.99	79.08	381
Mean				381.3
Average deviation				0.18 per cent
0	15.60			
20	11.07	4.53	22.39	818
31	8.75	6.85	33.83	821
41	6.82	8.78	43.42	822
50	5.24	10.36	51.24	820
60	3.69	11.91	58.90	817
70	2.30	13.30	65.78	817
Mean ..				819.2
Average deviation				0.22 per cent

Table II includes data obtained by hydrolyzing sucrose with live yeast cells. The values of N calculated therefrom are seen to have the same constancy as those obtained when using an invertase solution.

Two suggestions may be made as to sources of error in running hydrolyses with the live cells. The first is with regard to possible fermentation during the course of the hydrolysis which would affect the rotations, and the second has to do with the question of whether the yeast dies during the time of the hydrolysis.

To eliminate the first possibility, the buffer solutions used were saturated with toluene (1, 6), there being, however, no excess toluene in the hydrolysis mixture. Under these conditions no gas production was observed during the course of the hydrolysis. Furthermore, the hydrolyses were allowed to run to completion and the total change in rotation was found to be in agreement with the correct value for complete inversion of a 10 per cent sucrose solution, namely 20.22°. An

TABLE II
Hydrolyses Using Live Yeast Cells (1 Gm. Yeast)

Time	Rotation	Δ Rotation	Per cent hydrolyzed	$N \times 10^3$
<i>min.</i>	<i>degrees</i>			
0	15.55			
30	9.99	5.56	27.50	678
40	8.33	7.22	35.71	676
55	5.99	9.56	47.28	677
70	3.96	11.59	57.32	675
85	2.21	13.34	65.97	676
100	0.72	14.83	73.34	679
Mean				676.8
Average deviation				0.18 per cent
0	15.58			
30	10.08	5.50	27.20	670
41	8.23	7.35	36.35	672
56	5.97	9.61	47.52	669
71	3.97	11.61	57.42	667
86	2.21	13.37	66.12	670
106	0.36	15.22	75.27	671
Mean				669.8
Average deviation				0.18 per cent

extreme idea that the invert products might be fermented in such a ratio as to still give constant values of N in spite of fermentation may be refuted by the fact that similar results are obtained when the amount of yeast and consequently the time required for hydrolysis is varied. The possibility of having such compensating errors in more than one case would seem to be extremely unlikely.

With regard to the second possible error, no change in ratio of live

to dead cells, as determined by staining with 0.005 per cent solution of methylene blue according to the staining method of Fraser (7), was found to occur during the course of the hydrolysis. It was, of course, impossible to eliminate the presence of some dead cells in the suspension, but the yeast before being used was repeatedly washed and microscopic examination of the suspension revealed only intact cells. In addition, the ratio of live to dead cells was extremely large, namely, 52 to 1.

In order to be assured that in using live cells the inverting action is confined to the cells and is not exhibiting itself extracellularly, samples of hydrolyzing mixtures were filtered cell-free without the addition of alkali. On being polarized, these samples failed to show any noticeable enzymic activity.

SUMMARY

1. A method is given whereby the course of hydrolysis of sucrose by live yeast cells may be followed with precision equal to that found when invertase solutions prepared from autolyzed yeast are used to cause inversion.

2. The practical value of the equation of Nelson and Hitchcock as a means of following the course of enzymic hydrolysis of sucrose is hereby extended.

3. The inversion of sucrose by live yeast cells and by extracted invertase has been quantitatively compared.

4. The course of hydrolysis of sucrose by the invertase of Fleischmann's yeast has been found to be identical *in vivo* and *in vitro*.

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THE TEMPERATURE CHARACTERISTIC OF RESPIRATION OF AZOTOBACTER

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(Accepted for publication, January 25, 1932)

I

This paper will present data on the rate of respiration of *Azotobacter vinelandii* as a function of temperature over a wide physiological range. The value of μ , the critical thermal increment or temperature characteristic, will be calculated for the temperature range over which studies on *Azotobacter* are generally conducted, 20–30°C.

II

The Warburg technique employed to measure rate of oxygen consumption has been described in complete detail elsewhere (1, 2). The culture medium consisted of 1 per cent glucose dissolved in the clear liquid obtained after the following mixture has been thoroughly shaken, allowed to stand, and settle: 0.8 gm. K_2HPO_4 , 0.2 gm. KH_2PO_4 , 0.2 gm. NaCl, 0.2 gm. $MgSO_4 \cdot 7H_2O$, 0.1 gm. $CaSO_4 \cdot 2H_2O$, 0.01 gm. $Fe_2(SO_4)_3 \cdot 9H_2O$, 1000 gm. H_2O . No fixed nitrogen was added. The cultures employed in the Warburg vessels were first grown in an air thermostat at 28°C. in sterilized, aerated, 250 cc. gas wash bottles containing 50–100 cc. of nutrient medium. The temperature characteristic has been obtained from two different values, R_1 and R_2 , of the rates of respiration at two corresponding absolute temperatures, T_1 and T_2 , by means of the customary Arrhenius function,

$$\mu = 1.99 (T_1 T_2 / (T_1 - T_2)) \log_e R_2 / R_1 \text{ cal.} \quad (1)$$

III

Fig. 1 shows the effect of temperature upon rate of respiration over the temperature range 15–43°C. In Curve I the rate is plotted directly against the temperature, in Curve II the logarithm of the rate is plotted against the reciprocal of the absolute temperature. Triplicate simultaneous determinations were run at each temperature

investigated, proceeding from the lowest to the highest temperature. At each new temperature fresh aliquot portions were drawn as needed

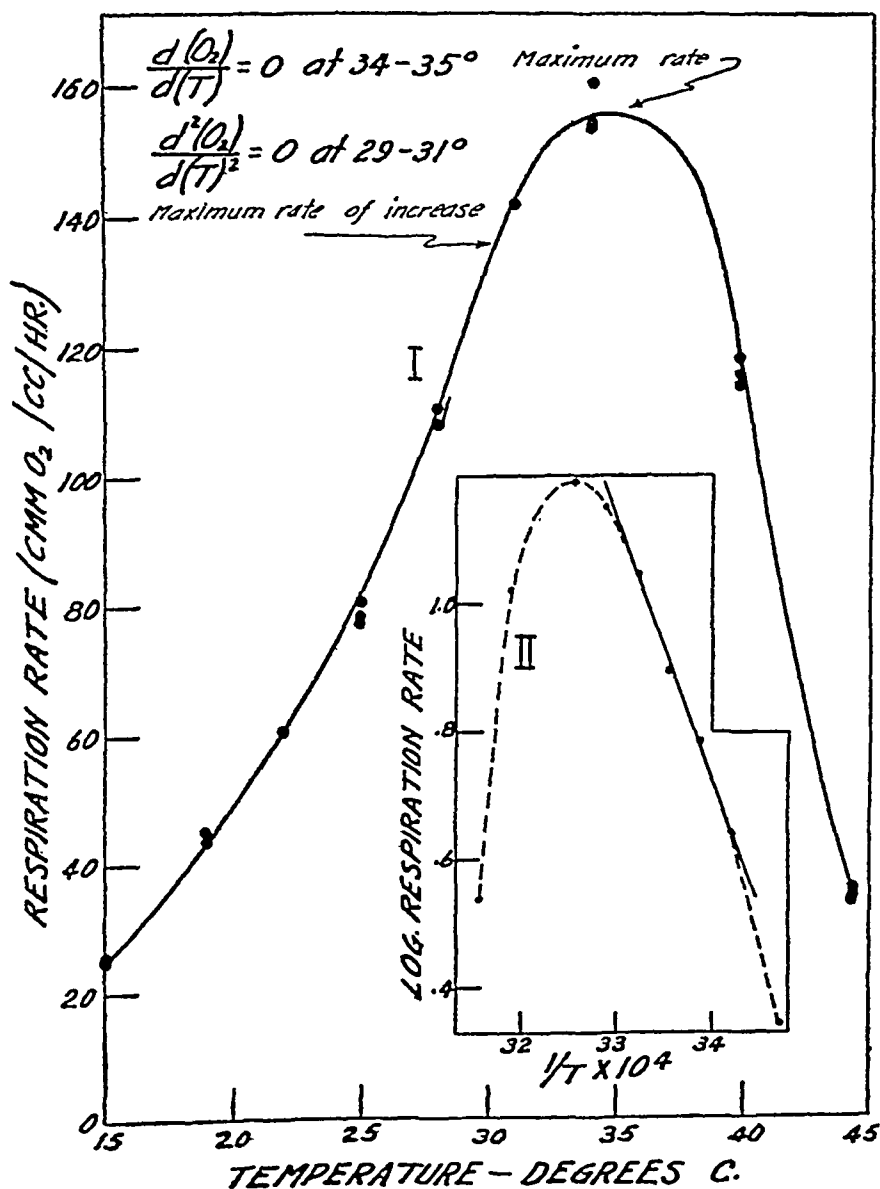


FIG. 1

from the stem culture kept in the ice chest at 10°C. during the $13\frac{1}{2}$ hours required to perform the experiment. A 3 day culture was em-

TABLE I
Temperature Characteristic of Azotobacter Respiration

Exp. No.	Age of culture	Respiration rate c.mm. O ₂ /cc /hr.		No. of determinations	Temperature		μ (cal.)
		R_1	R_2		T_1	T_2	
	days				°C.	°C.	
I (a)	3	29.3	40.3	3	19	22	18,200
(b)	3	40.3	52.7	3	22	25	15,600*
(c)	3	52.7	73.3	3	25	28	19,800
II (a)	3	19.9	28.2	5	24	27	20,650
(b)	3	28.2	35.9	5	27	30	19,275
III† (a)	3	17.8	33.0	4	24	30	18,400
(b)	3	19.1	37.1	4	24	30	18,900
(c)	3	19.8	37.0	4	24	30	18,750
IV (a)	2	32.9	64.5	4	23	29	20,000
(b)	2	33.5	63.0	3	23	29	18,750‡
(c)	2	27.1	53.5	3	23	29	20,250§
V (a)	2	38.2	64.5	4	23	29	15,550*
VI (a)	4	36.9	51.9	4	23	26	20,050
(b)	4	51.9	74.3	4	26	29	21,500
VII (a)	4	90.5	48.9	3	29	23	18,250
(b)	7	31.8	17.2	3	29	23	18,250
VIII (a)	4	21.5	40.7	3	23	29	18,950
Mean.....							19,333* \pm 165¶

In any one experiment (i.e., Experiment I, Experiment II, etc.) a given culture of a given age was placed in a thermostat at 10°C.; after 30–40 minutes aliquot portions were removed and measurements were commenced, generally with the lowest temperature first. Three to four determinations (i.e. involving three to four respiration vessels) were carried out, the manometer readings being taken at 10 minute intervals over a period of 40–60 minutes. At each different temperature fresh aliquot portions were used. As shown in Table II such aliquot portions maintained a constant respiration capacity over a long period of time.

The pH of all cultures was 6.7 ± 0.2 except VII (b), pH 6.0.

Temperatures are accurate to $\pm 0.02^\circ\text{C}$.

* All the experiments performed at 21 per cent oxygen are reported in this table.

ployed. The temperature function represented in Fig. 1 is quite typical of many biological processes, and its chief points of interest are its substantially symmetrical character, the maximum occurring at 34–35°C., and the suggested positions of the upper and lower limits of respiration at about 50° and 10°C. The value of μ appears to be constant between 20° and 30°C., in this single experiment. A critical temperature at about 19°C. is suggested.

Table I summarizes the experimental data obtained from a considerable number of experiments showing that respiration of *Azotobacter* possesses a constant temperature characteristic of $19,330 \pm 165$ over the range 20–30°. This value is not one of those commonly obtained for respiration in various other organisms. The customary values given by Crozier (3) are approximately 11,500, or 16,100 to 16,700, with occasional values (for winter frogs) of 21,600 to 24,000. Tang (9) has found μ values of 13,100 and 21,050 for the rate of oxygen consumption of *Zea mays* above and below 19.5°C., respectively. Recently Crozier has indicated (4) that in the case of respiration of yeast less frequently observed values are obtained, and that the mechanism of respiration is sufficiently complex so that one of a number of temperature characteristics may be found according to the prevailing metabolic condition characteristic of the experimental conditions. Although the value of 19,330 for *Azotobacter* is somewhat atypical, its order of magnitude is that of oxidation processes as compared to hydrolytic ones.

Table II indicates that the changes in respiration rate consequent

The two starred values were not used in calculating the mean μ since they are obviously too far removed from this mean.

† See also Table III.

‡ Measured in 100 per cent O₂.

§ Glucose added to 1 per cent. In all other cases the glucose content ranged from 0.3 to 0.8 per cent, depending upon how much of the initial 1 per cent had been consumed during the growth of the culture previous to use.

|| In this experiment the higher temperature was studied first. In all others the lower temperature was studied first.

¶ The probable error of the mean has been calculated by means of the usual formula $P.E._{mean} = \pm 0.6745 (\Sigma(v^2)/n(n-1))^{1/2}$. The probable error of any single value calculates to be ± 675 cal. (i.e., ± 3.5 per cent) from the formula $P.E._{single} = \pm 0.6745 (\Sigma(v^2)/(n-1))^{1/2}$.

upon altering the temperature of a culture cyclically from a relatively high, normal temperature to 10°C. are satisfactorily reversible over a long period of time (at least 2 to 4 days), and that the method em-

TABLE II
Constancy of Respiration Rate of Cultures Maintained at 10°C.

Time in ice chest	Respiration rate c.mm. O ₂ /3 cc./hr. at 28°C.			
	0 hrs.*	24 hrs.	48 hrs.	96 hrs.
Diluted† 1½ times (air‡).....	59.5	60.0	63.6	56
Diluted 1½ times (Ca. 21 per cent O ₂ in H ₂ ‡).....	69.5	69.5		
Undiluted (air‡).....	100	109		

Culture 1 day *A. chroococcum* Strain SM 1.

pH 6.7 ± 0.2.

Respiration rates were measured over a period of 40 to 60 minutes immediately after establishment of thermal equilibrium (i.e. in ca. 10 minutes) after removal from ice chest.

* Zero hour culture was also cooled to 10°C. during a period of 30-45 minutes before use.

† This refers to dilution of the culture with fresh medium.

‡ This refers to the gas mixture obtaining during the experiment; previous to the experiment air always obtained.

TABLE III
Effect of Volume of Culture on μ of Respiration Rate

Culture per vessel	No. of det.	Temp.	Rate resp. c.mm. O ₂ /hr.	μ (cal.)
cc.				
1*	4	24	17.8	18,400
	4	30	33.0	
2	4	24	38.1	18,900
	4	30	74.1	
3	4	24	59.3	18,750
	4	30	111	

3 day old *A. vinelandii* undiluted. 21 per cent O₂ in N₂.

* The same pipettes, respectively, 3, 2, and 1 cc. were employed at each temperature, but it is probable that the 1 cc. pipette employed contained slightly less than ½ and ⅓ respectively the volumes of the 2 and 3 cc. pipettes.

1.0 atmosphere O_2 is perfectly linear. (3) For short periods of time (1 to 5 hours) changes in rate induced by sudden changes in oxygen tension are immediate and reversible. (4) The rate may become enormously high in very young cultures (6–20 hours old), attaining a value of 5000 c.mm. O_2 /mg./hr. at 28°C., i.e., *Azotobacter* may burn seven to eight times its own dry weight of glucose per hour completely to CO_2 and H_2O . This rate of energy consumption is possibly as high as or higher than that shown by any other living organism. In the experiments reported in Table I the rate of oxygen consumption probably ranges from 100 to 500 c.mm./mg. of dry matter/hr. It is hardly possible, however, that respiration in *Azotobacter* depends upon more than one mechanism (in which case an unusual μ value might be expected) in the sense implied by Elvehjem (6), who found that with bakers' yeast respiration depends upon two mechanisms, one KCN-labile, the other KCN-stabile.

In the investigation of biological temperature characteristics it is often desirable to have the measurements of reaction velocity so numerous that the variation in velocity at constant temperature may itself be investigated. This is true chiefly where a single organism (say, its heart beat rate) is being investigated. Presumably no such measurements of variation are possible in the case of *Azotobacter*: (1) the observed minor variations in any given experiment are unquestionably owing solely to the method or technique of respiration measurement; (2) ordinarily the number of organisms dealt with are 50 to 150 million (per cc.); (3) the cultures employed in the experiments reported were transferred daily for a long period of time before use, thus eliminating greatly or completely the possibility of the existence of several metabolic types of individuals or their quantitative importance compared with the predominating type.

SUMMARY

The temperature characteristic of respiration of *Azotobacter vinelandii* possesses a constant value of $19,330 \pm 165$ over the temperature

from the far less important ratio of volume of gas to volume of liquid) was critically low and any resulting lessened removal of CO_2 or establishing of respective equilibrium concentrations of O_2 (at different O_2 pressures) in all local parts of the culture medium would result in a raised maximum, not a lowered maximum as Fife believed.

range 20–30°C. This value is independent of pH, oxygen tension, age of culture, and other factors within the limits studied.

The optimum temperature of respiration is 34–35°C., with limits at about 10° and 50°C.

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THE SPECTRAL SENSIBILITY OF THE SUN-FISH AS EVIDENCE FOR A DOUBLE VISUAL SYSTEM

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INTRODUCTION

Most fish, like most other vertebrates, possess an anatomically duplex retinal system, composed of rods and of cones (Schultze, 1866; Wunder, 1925). The Duplicity Theory, proposed in 1894 by von Kries, postulates that animals which have such an anatomically double retinal system show this physiologically by the presence of two types of photosensory behavior. This hypothesis is based, in part, on the work of Schultze (1866), Hering and Hillebrand (Hillebrand, 1889), and of Koenig (1894), and also follows a suggestion made by Parinaud in 1881 (see Tschermak, 1929). According to the Duplicity Theory, which is applied to all vertebrates, the rods are responsible for vision at low intensities of illumination while the cones are functional at the higher intensities. Subsidiary to this hypothesis is the further one that cone vision in the lower vertebrates may also be accompanied by color perception.

Tschermak (1929) justly observes that "decisive weight must be given only to physiological arguments (in favor of the Duplicity Theory), especially those indicating different visibility functions in dim and in bright vision, and not to histological data, since the findings of comparative anatomy are not always unequivocal . . ." It was, however, precisely on the physiological arguments that opposition to the Duplicity Theory was maintained for many years by von Hess (general summary, 1922). Hess' attack was along two lines. He believed that the lower vertebrates and especially fish possessed, at all intensities, the same spectral sensibilities as those found in totally

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color-blind humans and in normal human dim vision. Furthermore, Hess maintained that the lower vertebrates showed an inability to discriminate colors.

There is evidence (Grundfest, 1932) showing that the sensibility of *Lepomis* to very dim spectral lights is determined by the absorption spectrum of its own visual purple and therefore does not correspond to the visibility curve of human color-blinds. This is entirely contrary to Hess' first argument. His second position has been made untenable by the evidence of Sumner (1911), Mast (1914), and especially of von Frisch and his students (review by von Frisch, 1925). These investigators have presented proof in a number of different ways that fish can discriminate colors.

These two types of evidence, while they remove the objections of Hess, do not settle the problem raised by the Duplicity Theory as to the presence of a dual photosensory system. However, von Frisch (1924) has shown that color discrimination in fish is possible only at intensities that are well above the threshold for brightness perception. Furthermore, Bauer (1910) has found that fish exhibit the Purkinje phenomenon. Since Koenig and Ritter (1891) have shown that this effect, in man, is due to the difference in the spectral sensibilities of the rods and cones, the results of von Frisch and of Bauer are strong evidence to show that two different retinal mechanisms also occur in fish. Up to the present, however, no quantitative evidence has been available. It is my purpose in making the present measurements to supply this lack, and to show quantitatively that in the case of the sun-fish *Lepomis* the visibility function at high spectral intensities is different from that obtained for low, thus affording direct proof that the eye of the sun-fish is a duplex mechanism in the sense postulated by the Duplicity Theory.

Method

In a previous paper (Grundfest, 1932) I have reported measurements on the sensibility of the sun-fish to very dim monochromatic lights. Those data were obtained by using as an index the "rheotropic" response of an individual animal to an object moving in its visual field. Measurements were made of the relative energies of various spectral beams which just enable the animal to discriminate a pattern composed of alternate bars and spaces. Such discrimination is evidenced by the orienting response of the fish to a movement of the pattern. In

the present work the spectral visibility function at higher intensities has been determined in the same way by a suitable modification of this method.

The pattern used for measuring the dim-visibility function was composed of rather large vertical bars and spaces, since it was desired to study the vision of the animal as close to the threshold intensities as possible. The present measurements however are concerned with a brightness level several hundred times above the threshold. A pattern, to be just discriminable at these intensities, must be composed of very fine elements, as has been shown for the human eye by Koenig (1897). This relation between light intensity and visual acuity has been further developed by Hecht (1928) and extended to apply to all visual systems composed of a large number of unitary receptors (Hecht and Wolf, 1929).

Koenig pointed out that the relation between visual acuity and intensity involves two functions. At low visual acuities and the correspondingly low intensities, discrimination is accomplished by the rods, while at the higher acuities it is performed by the cones. The use in these experiments, of a fine pattern corresponding to a high visual acuity makes possible, therefore, measurements of the sensibility of the sun-fish to spectral lights of intensities far above the threshold. Since the data are obtained in the same way as those for very dim illuminations, the results of the two measurements are strictly comparable and it is possible to decide whether the visual system of *Lepomis* is composed of two functional entities.

Apparatus

The experimental arrangement is shown diagrammatically in Fig. 1, and is a modified form of the apparatus described in my previous paper (Grundfest, 1932). Light from a 500 watt concentrated-filament lamp illuminates a piece of opal glass, thus furnishing an evenly radiating source of light. By means of a lens the light from the opal plate, after passing through 4 cm. of water in a cell, is focussed on a neutral wedge so that its intensity may be varied continuously. Beyond the wedge the light spreads out, being helped in this process by a negative lens, and is reflected upward by a mirror so as to fall on the inside of a hollow, truncated 45° cone made of plaster of Paris. From here the light is diffusely reflected inward through the movable screen pattern toward the fish which is in a cylindrical glass jar. The glass jar, the screen pattern, and the plaster of Paris cone rest on a glass-topped table as shown in Fig. 1.

The movable pattern in the previously mentioned work (Grundfest, 1932) was made of a cylinder composed of rather large metal bars. These gave a very low visual acuity, and made the minimal illumination at which the fish could discriminate the moving pattern also

very low. The movable patterns in the present experiments consist of fine wire in order that the visual acuity and the corresponding illu-

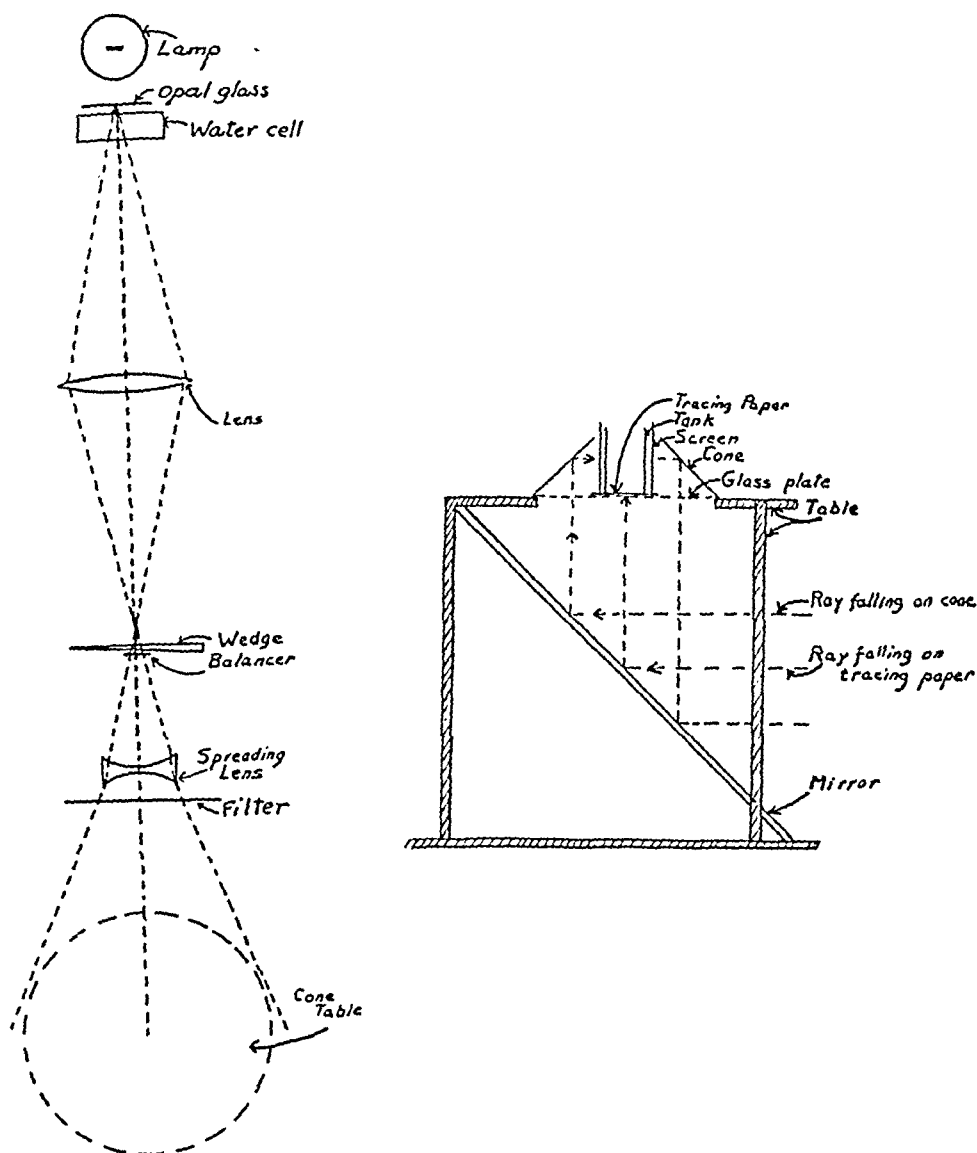


FIG. 1. Diagrammatic arrangement of the apparatus.

minations should be much higher than the threshold. Two screens have been made by taping vertically on the outside of a hollow glass cylinder lengths of fine wire spaced about 6-8 mm. apart. The di-

ameter of the wire used for Screen A is 0.2 mm. and for Screen B it is 0.1 mm. Discrimination of these patterns corresponds to visual acuities of 0.06 and 0.12 Snellen units respectively.

A spectroscope used as a source of monochromatic radiation does not deliver the high intensities required for the discrimination by the fish of these patterns. Wratten monochromatic filters Nos. 71A to 76 have therefore been employed in conjunction with the 500 watt lamp. They are inserted in the path of the light after the neutral wedge and the spreading lens. The transmissions of these filters were measured with a Koenig-Martens spectrophotometer. The energy distribution of a 1,000 watt lamp was determined by means of a Hilger constant deviation spectrometer, a Hilger linear thermopile, and a Leeds and Northrup type HS galvanometer. The 500 watt lamp used was so similar in color temperature to the 1,000 watt lamp, that I considered the energy distribution in the spectrum of the two as identical for the present purposes.

The relative energy transmitted by the filters in conjunction with the lamp has then been calculated in the following way. The transmission of each filter at any wave-length was multiplied by the relative energy emitted by the lamp at that wave-length. These values were plotted for each filter and the relative areas of each curve measured planimetrically. These areas correspond to the relative total energy obtained from the lamp and the filter. The abscissa at the center of gravity of each curve has been taken as the wave-length of the beam. Table I gives the data for the six filters.

In these calculations, it has been arbitrarily assumed that wave-lengths longer than 700 m μ are not effective in the vision of *Lepomis*. The areas and centers of gravity of filters 71A and 72 have accordingly been calculated on that basis. The visibility function of one animal (5NS) has been determined at very low illuminations with the present arrangement but using the large stripes corresponding to the low visual acuity. This served to test the last assumption as well as the general permissibility of using the rather broad spectral beams obtainable with filters.

The results are shown in Table II. Included in Table II, in the last column, are comparable values taken from my previous work with *Lepomis* (Grundfest, 1932). The values are secured from the curve

representing the average of thirteen animals, measured with narrow spectroscopic bands, and not with broad filters. It is evident that the results obtained from Animal 5NS by means of Wratten filters resemble closely those previously gotten with the more elaborate apparatus using a spectrometer. The wave-length of maximum effectiveness is near 540 $m\mu$, about where it was found previously; more-

TABLE I

Energy transmission of six Wratten filters in conjunction with a 500 watt, concentrated-filament Mazda lamp.

Filter No.	Central wave-length <i>mμ</i>	Relative energy transmitted
76	451	1.00
75	491	2.05
74	533	2.05
73	577	2.38
72	612	1.33
71A	660	5.33

TABLE II

Relative effectiveness of spectral light of very low illuminations as furnished by Wratten filters and by a spectrometer.

Wave-length <i>mμ</i>	Log relative energy. Animal 5NS	Relative effectiveness	
		For 5NS with Wratten filters	For 13 animals with spectrometer
451	0.113	22.8	
491	1.947	33.3	25.0
533	1.578	78.0	81.0
577	0.282	15.4	11.5
612	0.523	8.9	5.0

over, the effective spectral portion seems to be quite narrow, as before. The small number of points at which determinations of such a narrow curve could be made with the present arrangement prevents accurate conclusions; but judging by the agreement of the two results and remembering the range of individual variation possible, it seems extremely likely that the use of filters and the neglect of wave-lengths longer than 700 $m\mu$ are both legitimate procedures.

The control of intensity is effected by an Eastman Kodak balanced neutral wedge which is 20 cm. long and 3 cm. wide. It has a range of 1:1,000. The wedge, securely mounted in a movable, metal carriage has been calibrated photometrically, in terms of the light transmitted at different parts of the wedge corresponding to different positions of the engraved millimeter scale attached to the mounting of the wedge itself. The relation is such that the logarithm of the light intensity transmitted is a linear function of the distance along the wedge. The position of the scale can be read accurately to 0.1 mm., though in these measurements the readings have rarely been to closer than 1 mm.

Procedure

The animals were kept alive in individual, balanced tanks. They have been obtained from the New York Aquarium, through the courtesy of Mr. Breder.

The experimental procedure followed in the present work is very similar to that already described in my study of the spectral sensibility of the sun-fish at low illuminations (Grundfest, 1932). At the beginning of each experiment, a fish is placed in a clean cylindrical jar which is filled with 250 cc. of filtered tap water. The jar is 9 cm. in inside diameter and the depth of the water is about 4 cm. This tank, containing the animal, is then placed on the cone table in the position shown in Fig. 1, and the fish is allowed to become dark adapted for about 2 hours. At the end of this time measurements are begun.

One of the Wratten filters is placed in the path of the light beam, the wedge is set at its densest point, and a shutter is opened, permitting the light beam to fall on the mirror. The hollow glass cylinder which carries the fine vertical line pattern is rotated in either direction. If the animal does not respond, the intensity of the beam is increased somewhat by moving the wedge out about 5 mm. and the cylinder is again rotated. This procedure is repeated until the animal gives a definite orienting reaction. The light is again dimmed by moving the wedge in a few mm. By gradual search the setting of the wedge is determined at which the animal just responds. This scale reading is recorded to the nearest mm. on the wedge scale and the measurement repeated. Then another set of measurements is made for a different Wratten filter in the same way, and the process con-

tinued until all the filters have been tested. There is no regular order in which the filters are used. The time required for a complete experiment is about $1\frac{1}{2}$ hours, exclusive of the adaptation period.

The readings secured are transformed into energy values by the calibrations. These values—as $\log E$ —are plotted against their respective wave-lengths, and a smooth curve is drawn through them in order to secure the most effective wave-length. The reciprocal of the energy corresponding to this minimum is given a value of 100 per

TABLE III

Relative energy ($\log E$) and relative effectiveness (E_{eff}) of spectral lights of high intensities for stimulation of *Lepomis*. Data on four animals—3NS, 4NS, 5NS, and 6NS.

Wave-length <i>mμ</i>	Screen A								Screen B	
	3NS		4NS	5NSI	5NSII	5NSIII	6NS		6NS	
	$\log E$	E_{eff}	$\log E$	$\log E$	$\log E$	$\log E$	$\log E$	E_{eff}	$\log E$	E_{eff}
451	2.303	9.9	2.351	2.747	2.909	2.767	2.949	12.6	3.091	14.4
491	2.191	12.9	2.413		2.877	2.797	2.453	39.5	2.675	37.6
533	1.383	82.6	2.271	2.635	2.716	2.777	2.312	54.7	2.494 2.433	57.0 65.6
577	1.343	90.6	1.670	2.195	2.256	2.276	2.114	86.3	2.316	85.9
612	2.043 1.760	18.1 34.1	1.745		2.124	2.083	2.023	106.4	2.285	92.3
660	2.828	3.0	2.754	3.131	3.050	3.151	2.989	11.5	3.212	10.9

cent, and the relative percentage effectiveness of the other parts of the spectrum are calculated from it by dividing the energy at the maximum by the energy required at the other wave-lengths.

RESULTS

In order to make these measurements of quantitative significance, it is necessary to observe one important precaution. Koenig and Ritter (1891) have obtained visibility curves intermediate in shape between that of the rods and that of the cones, merely by using an

intermediate brightness level in their measurements. This effect, indeed, is the basis of their explanation for the Purkinje phenomenon. It is therefore important to ascertain that the measurements with

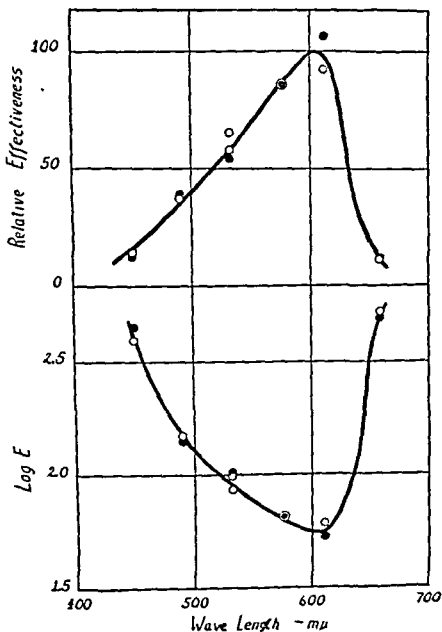


FIG. 2. Cone visibility function for Animal 6NS determined at two brightness levels. The solid circles are for a visual acuity of 0.06; the clear circles for a visual acuity of 0.12. In the former case the points have been raised 0.2 units on the log E ordinates to permit superposition of the two sets at 577 mμ.

Lepomis are made at an intensity level which assures the determination of the cone curve alone and not of an intermediate condition.

It is for this purpose that the pattern of Screen B was made. The

visual acuity corresponding to it is twice that of Screen A, and therefore demands still higher illuminations in order to be resolved by the animal's eyes. Animal 6NS was measured with both screens. The

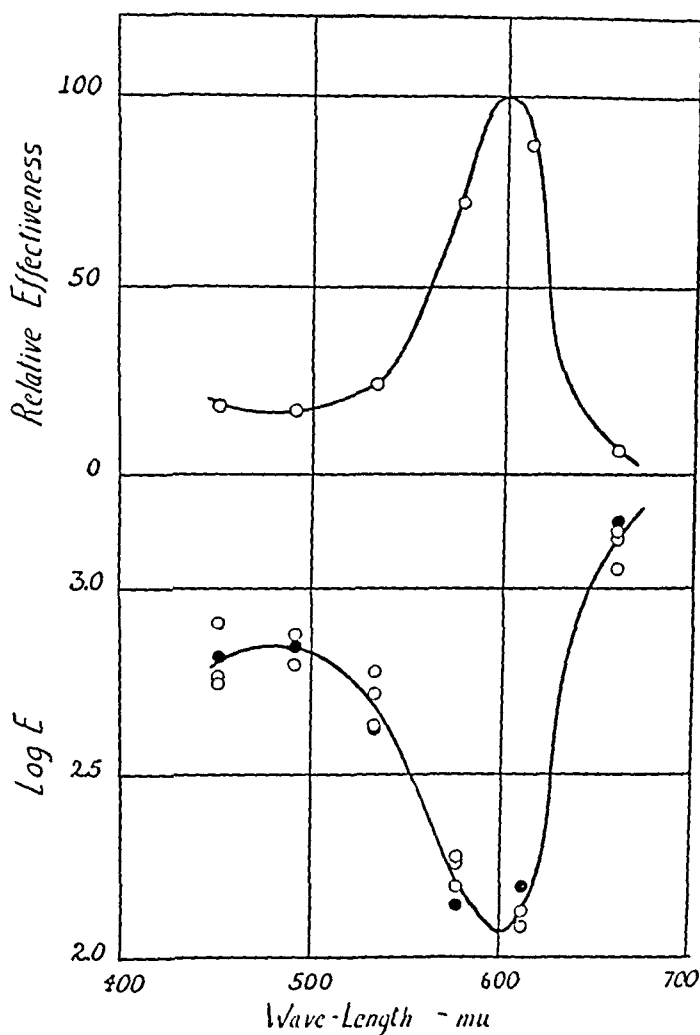


FIG. 3. Cone visibility functions of Animal 5NS (clear circles) and 4NS (filled circles). The latter values are raised by 0.45 unit on the log E ordinates. The upper figure is obtained from the average log E curve.

data are given in Table III, and are plotted in Fig. 2. The curves obtained in both cases are identical if allowance is made for the higher intensity level in the measurements with the finer screen. This is shown in Fig. 2 where a constant is added to the values of log E for

Set A (filled circles). This constant is so chosen that one point in each set (double circle, 577 $m\mu$) becomes identical; its value is 0.2 unit on the log E scale. The clear circles are the values obtained with Screen B. Both sets of data give the same visibility curve, showing

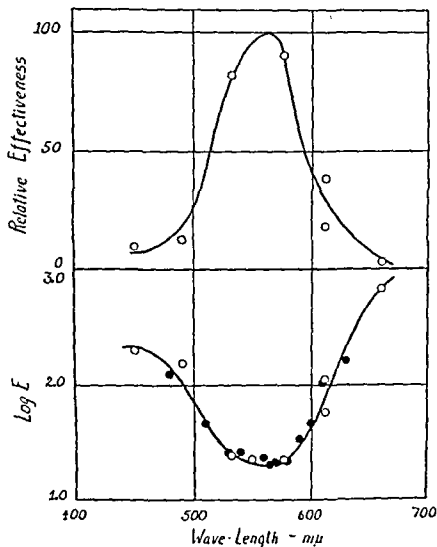


FIG. 4. Cone visibility (clear circles) of Animal 3NS. The solid circles are the data for Animal 8 (Grundfest, 1932, Fig. 6, lower half) shifted 30 $m\mu$ toward the red.

that the measurements made with Screen A represent the cone visibility function and not an intermediate condition.

Using Screen A only, I measured three other animals in addition to Animal 6NS. The measurements are all given in Table III. Several sets of measurements have been made with Animal 5NS. They are shown in Fig. 3 together with the data for 4NS (which have been raised 0.45 unit in the figure). This curve is much narrower than

that for 6NS and probably represents a condition comparable to that found in most measurements on the rod visibility function. The data for Animal 3NS are of particular interest and will be discussed fully in the following section. They are plotted in Fig. 4 (clear circles). The visibility curve for this animal is much broader and more regular than those of the other animals.

In every case, the wave-length of maximum efficiency at high illuminations is further to the red than is the corresponding maximum for vision at low illuminations. The previous work with *Lepomis* showed the dim vision maximum to be at $540\text{ m}\mu$. As can be seen from the figures the maximum for high illuminations does not occur at the same wave-length in all the animals. In the case of Animal 3NS it is $30\text{ m}\mu$, and in the case of the other animals it is $60\text{ m}\mu$ further toward the red than is the maximum for the rod visibility function. The reason for this difference will be discussed later. At present, it is important only to note that the difference between the visibility curves at the two intensity levels clearly shows that there are two photosensory systems in *Lepomis* each having a different sensibility to spectral lights. One of these systems is responsible for dim vision and is presumably composed of the rods. The other system is predominantly active in bright illumination, and probably is composed of the cones. It will be recalled that a similar difference in the position of the maximum of the cone and of the rod visibility curves is also found in human vision.

DISCUSSION

In comparing the visibility curve of *Lepomis* at low illuminations with the absorption spectrum of its visual purple, it was found (Grundfest, 1932) that the visibility curve is very much narrower than the absorption curve. To account for this difference in the shape of the two curves I have suggested the hypothesis that the eye of *Lepomis* contains, in addition to visual purple, two light-absorbing, but not light-sensitive, pigments. These act as spectral filters and distort the shape of the visibility curve out of agreement with the form demanded by the absorption spectrum of the visual purple. One of these pigments is not identified but it is suggested that the second may be carotin. The first is assumed to be relatively fixed in quantity while

the concentration of carotin is known to vary with the physiological condition of the animal. Among the evidence presented for this hypothesis have been the visibility curves of one animal (No. 8) which were taken several weeks apart. The first of these curves 8I closely approached on the short wave side the curve predicted from the absorption spectrum of fish visual purple. On the right side of the maximum it agreed with the curves found for the other animals. Two later measurements showed a gradual change in the left portion of the visibility curve until the entire function became similar to that obtained with the other twelve animals. From these data it was concluded that the visibility function at low illuminations tends toward agreement with that demanded by the absorption spectrum of the visual purple when the complications produced by the light absorbing pigments are eliminated.

This interpretation can also be applied to the data here given on the cone visibility function. Though a quantitative treatment of the variations in the visibility function is not possible, certain points may be cleared up. For example, in one case we can compare the rod and the cone visibility curves in an approximate way. This comparison can be made between the data for Animal 3NS (cone visibility) and for 8I (rod visibility) given in full detail in my previous paper. The comparison is shown, graphically, in Fig. 4. The clear circles represent the cone function data of 3NS while the filled circles are the rod data of 8I. In the latter case, the wave-lengths have been increased by 30 $m\mu$ while the two arbitrary log E scales have been made equal by adding 0.6 unit to the rod values. As mentioned above the data for Animal 8I have been found to approach the theoretical rod visibility function on the short wave-length side of the maximum. Remarkably good agreement is evident between the two sets of data, considering that, in one case, broad filter bands and, in the other, very narrow spectral bands have been used.

This agreement must not, however, be pressed too closely. I believe it indicates clearly that the undistorted cone curve is similar to the rod curve but shifted toward the red. The magnitude of the shift cannot be safely stated from these measurements, since it is assumed that there is still present one pigment which distorts the long wave portions of both curves. The absorption spectrum of this pigment most

likely does not change in the two cases and an error is introduced by moving the rod curve to coincide with the cone data. The shift is certainly 30 $m\mu$ and most probably greater, because the remaining pigment would tend to distort the point of maximum efficiency, moving it toward the short wave end. Unfortunately the rod function of 3NS, which could have furnished material for an adequate comparison, was not determined.

The foregoing indicates that the cone visibility function of *Lepomis* is similar to its rod function but shifted toward the red. The rod curve is determined by the absorption spectrum of visual purple. It therefore seems likely that the form of the cone curve is also fixed by a photosensitive substance whose absorption spectrum is similar to that of visual purple but displaced some 30–60 $m\mu$ toward the red. As I have mentioned above, this same relation occurs also between the rod and cone systems of man; Honigsmann (1921) finds a similar behavior in the case of the fowl.

The visual purple found in fish, however, is different from that of the hen and the human (Koettgen and Abelsdorff). We are therefore confronted with an interesting situation. On the one hand, we know that rod vision is mediated in fish by one kind of visual purple and in the other vertebrates by a second type. On the other hand, the cone visibility curve of each group is similar in form to its particular rod curve and both types of cone curves are displaced in the same direction relative to their rod curves. Is there any relation between the sensitive substances responsible for rod and for cone vision, which would account for the similarity in the shapes of their visibility curves and also for the similar displacement which each cone function undergoes with respect to its corresponding rod function? It is of course conceivable that these results are purely fortuitous. However, it must be remembered that the simultaneous occurrence of two coincidences is fairly rare. Yet this is exactly what must be assumed upon such a theory. According to this view we find two vertebrate groups having different visual purples. In each of these groups has arisen an independent cone photosensitive substance.¹ The first co-

¹ For simplicity, I have neglected the possibility of subdivisions in the sensitive substances. Such subgroups are presumably so closely related that they can be at present considered as behaving like an entity.

incidence arises from the fact that the absorption spectrum of each cone substance is similar to the absorption spectrum of the corresponding visual purple. The second coincidence concerns the fact that each cone curve is displaced along the spectrum with regard to its rod curve in an identical manner. Although such a series of chance occurrences is possible, it seems more likely that the appearance of identical phenomena in two different photochemical systems may be due to one and the same fundamental cause.

With relation to the similar shapes of the human rod and cone visibility curves, Hecht had suggested (Hecht and Williams, 1922) that visual purple is the common photosensory substance of both rods and cones. He explained the displacement of the cone curve toward the red end of the spectrum as due to a difference in the densities and refractive indices of the media in which the visual purple is dissolved. This is an application of Kundt's rule, which states that the absorption spectrum of a substance dissolved in different media is shifted toward the red in the medium of higher density. Even though Hecht (personal communication) no longer subscribes to this idea, the suggestion in my opinion still merits serious consideration. Such a hypothesis simplifies the problem as to why the different photochemical systems which are present in the eye of man and in fish, respectively, behave in a parallel manner. The answer, according to this hypothesis, is that there are only two different chemical substances which behave in a similar manner because of like relations between the solvent media in their respective rods and cones. The shift of the two cone curves is thus ascribed to a single physical factor, namely the greater density of the solvent media in the cones.

On first glance, there is a serious objection to the view that the photochemical substances of the rods and cones are identical. This arises from the fact that the Young-Helmholtz theory of color vision postulates three distinct cone substances having different absorption spectra, while visual purple is generally assumed to be a single entity. This objection disappears on closer examination. Hecht's modification of the trichromatic theory (Hecht, 1930) calls for three sensitive substances which have nearly identical absorption spectra and are closely related in chemical composition; they may perhaps even be derived in development from the same precursor. It is the sum of

their absorption spectra which determines the cone visibility function. There is no inherent difficulty in assuming that the rod sensitive substance is also the sum total of these three components. It is immaterial, for present purposes, whether these are assumed to be intimately mixed in each rod, or whether they are supposed to be separated among different rods, as is presumably the situation in the cones. Since the rods differ fundamentally from the cones in that their cerebral termini produce on stimulation an achromatic sensation of brightness, either type of distribution would give the same end result.

The above hypothesis may possibly be submitted to an experimental test. It is assumed that the rods contain, just as do the cones, the three modifications of visual purple. According to modern ideas (Hecht, 1930), color blindness is a condition in which one color sensitive substance is replaced entirely or in part by another. This would mean here that one modification of visual purple is replaced by another. In the case of color-blinds, therefore, the rod visibility curve should undergo modifications similar to those found for the cone luminosity curves. An adequate test of this point involves a series of measurements with a higher degree of precision than was possible in the older data of Koenig (1903). However, it is doubtful whether any significant results could be obtained, because the changes in the visibility function which are to be predicted in color-blinds from Hecht's *V-G-R* curves are so slight that they easily fall into the range of variability found for normal trichromatic individuals.

In conclusion, I wish to thank Professor Selig Hecht for his help and encouragement during this investigation.

SUMMARY

1. An extension of a previously described method makes possible the measurement of the visibility function of *Lepomis* at high intensities of spectral illumination. This is accomplished by determining the relative energies of various spectral beams which will just produce a visual orienting response by the animal to the movement of a pattern composed of fine lines.

2. The function so determined is different from that obtained with a pattern composed of wide bars and spaces at a lower intensity level.

3. This difference furnishes direct and quantitative proof that the eye of *Lepomis* is a physiologically duplex visual system and parallels the known anatomical distinctions between the rods and cones.

4. A comparison of the visibility curves of the two systems indicates that both functions are similar in shape but that the cone curve is shifted to the red.

5. It is suggested that this relation between the two systems, which is also found in the human and the fowl, indicates that the photosensory substance is the same in each case for the rods and cones. According to this hypothesis, the shift of the cone curve is due to a common physical cause which depends on differences in the properties of the solvent media in the cones and in the rods.

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DISSIMILARITY OF INNER AND OUTER PROTOPLASMIC SURFACES IN VALONIA. III

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The dissimilarity of the inner and outer surfaces of the protoplasm of *Valonia macrophysa* is strikingly demonstrated by the large P.D. across the protoplasm observed when the solution applied to the external surface has the same composition as the vacuolar sap.¹ While in some of the earlier experiments² the external solution was natural *Valonia* sap, in the later measurements with improved technic³ it was artificial sap made up according to the analyses of L. M. Van der Pyl.⁴ The use of artificial sap may be open to criticism on the ground that it possibly lacks some substance which, although present in natural sap only in traces, may still be supposed to exert a considerable effect on the P.D. (Such effects have been reported for artificial systems: thus, Beutner⁵ finds that the addition of 4 mg. of pilocarpine to 100 cc. of physiological salt solution causes a decrease of 57 mv. in the P.D. between this solution and a layer of nitrobenzol containing oleic acid.) Accordingly, it seemed worth while to carry out a few additional measurements using natural sap when our supply of *Valonia* permitted the sacrifice of a few hundred cubic centimeters of cells to provide a sufficiently large sample.

¹ Similar evidence for the asymmetry of protoplasm has been found in *Nitella* (Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927-28, 11, 391), in *Halicystis* (Blinks, L. R., *J. Gen. Physiol.*, 1929-30, 13, 223), and possibly in muscle (Osterhout, W. J. V., *Biol. Rev.*, 1931, 6, 390).

² Osterhout, W. J. V., Damon, E. B., and Jacques, A. G., *J. Gen. Physiol.*, 1927-28, 11, 193.

³ Damon, E. B., *J. Gen. Physiol.*, 1929-30, 13, 207.

⁴ Osterhout, W. J. V., *J. Gen. Physiol.*, 1922-23, 5, 225.

⁵ Beutner, R., *J. Pharmacol.*, 1927, 31, 305.

The cells available for this purpose were taken from a lot which had been kept at the (Bermuda) laboratory for more than a year; they had been stored out of doors, but shaded from direct sunlight, in large glass bottles containing more than ten times their volume of sea water; the sea water had been changed occasionally. Under these conditions the cells had grown to more than twice their volume when collected. They appeared healthy, although they were lighter green in color than recently collected cells, and were somewhat more sensitive to rough handling. 5 months later, practically all the unused cells from this lot were still in good condition. Several samples of sap were extracted at different times in order to provide fresh material for the P.D. measurements. After these measurements had been completed, L. L. Burgess (of this laboratory) kindly determined the concentrations of KCl and NaCl in the combined samples.

	Mols per liter	
	KCl	NaCl
This sample of natural sap.....	0.542	0.111
Proportions used in making artificial sap ⁴	0.517	0.090

A considerable variation is to be expected in the composition of different samples of sap, since, as Jacques and Osterhout have pointed out,⁶ the K ÷ Na ratio of the sap changes during the growth of the cell, and is easily affected by external conditions (e.g., by handling the cells, by changing the sea water, or by illumination). Comparison of measurements in which fresh sap was used with others in which the sap had been kept from 1 to 4 days after extraction showed no differences which could be attributed to changes in the sap on standing. The impaled cells used in the measurements of P.D. were taken from a more recently collected lot (collected November 14, 1930, measured January 21–February 18, 1931) which had been stored out of doors as described above, with change of sea water at least once a week.

Since the limited supply of natural sap did not permit the use of a flowing contact for applying solutions to the cell (as in earlier measurements with artificial sap³), a less wasteful method was adopted in which the cell was completely immersed in a small volume of solution. For this procedure it proved convenient to impale the cell from above while supporting it on a glass ring as shown in Fig. 1. Advantages of this form of support are that it adds very little to the depth of solution needed to cover the cell and that it is easily rinsed, thus reducing the danger of carrying over sea water into the natural sap when the external solution is changed. (The rinsing consisted in dipping the cell rapidly several times in a second beaker of natural or artificial sap before placing it in the sample to be used in the P.D.

⁶ Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1930–31, 14, 301.

measurement.) The external and internal solutions were connected with balanced calomel electrodes in the same manner as described in an earlier report.⁷

The E.M.F. of the system was measured by a compensation method using a simple type of potentiometer and a Compton quadrant electrometer. It proved most convenient to use this combination as a deflection potentiometer; *i.e.*, the principal part of the unknown P.D. was balanced by a known P.D. from the potentiometer while the remaining small portion of the unknown P.D. was estimated from the electrometer deflection. The electrometer was adjusted to give a deflection of 5 to

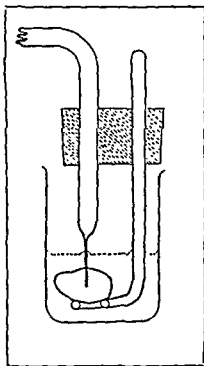


FIG. 1. Diagram showing the method of supporting a *Valonia* cell on a glass ring while electrical connection with the vacuole is established through a glass capillary filled with artificial *Valonia* sap. A doubly bored cork fastens the glass rod carrying the supporting ring to the tube on which the cell is impaled. The cell is first placed on the ring, and the rod is then pushed upward through the cork until the point of the capillary pierces the vacuole. This method was designed by L. R. Blinks.⁸

6 mm. per millivolt with the scale 4 feet from the mirror. The distance measured in practice, however, was the sum of the deflections to right and to left when the sign of the charge applied to the insulated quadrants was reversed (by means of a reversing switch in the electrometer circuit); since this total deflection amounted to 10 to 12 mm. per millivolt, the P.D. could easily be read to 0.1 millivolt. Since the time required for full deflection was somewhat less than 20 seconds, as many as

⁷ Osterhout, Damon, and Jacques,² p. 195.

⁸ Blinks, L. R., *J. Gen. Physiol.*, 1930-31, 14, 139; also unpublished experiments.

3 readings per minute were possible provided that the P.D. did not change so rapidly as to require a new setting of the potentiometer.

The conclusions reported in this paper are based on 28 measurements of P.D. using 13 impaled cells. Of these measurements, 12 were with natural sap, using 7 different cells, and 16 were with artificial sap, using 9 cells. The average duration of exposure to sap in each experiment was 90 minutes. The measurements were carried out at room temperature, which varied between 14° and $21^{\circ}\text{C}.$, but which in most cases was between 16° and $18^{\circ}\text{C}.$

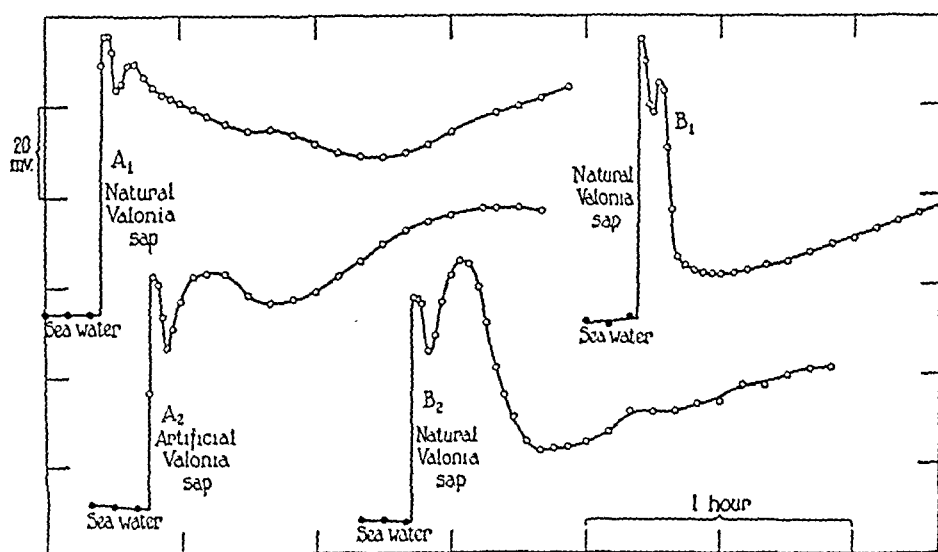


FIG. 2. Time curves showing changes in P.D. across *Valonia* protoplasm when the external solution is changed from sea water (shaded circles) to *Valonia* sap (open circles). The interior of the cell is positive in the external circuit with respect to both sea water and sap applied externally; *i.e.*, positive current tends to flow from the capillary through the measuring instrument to the solution bathing the outside of the cell. Curve A_1 represents a measurement using natural *Valonia* sap, Curve A_2 a later measurement on the same cell using artificial sap. Curves B_1 and B_2 represent similar measurements with natural sap on a second cell. To prevent confusion, the graphs are separated by a vertical and horizontal displacement.

Comparison of the P.D.-time curves with natural sap and with artificial sap fails to disclose any effects which can be ascribed to differences between the two solutions. Any such effects are evidently much smaller than the variations among individual cells measured with the same solution, or than changes in a single cell measured at different

times with the same solution. This is illustrated by the P.D.-time curves in Fig. 2, where Curve A₁, representing a measurement using natural sap, has the same general shape as Curve A₂, representing a later measurement on the same cell using artificial sap. Differences between these two curves are closely paralleled by differences between Curves B₁ and B₂, both of which represent measurements with natural sap on a second cell. Reasonably good agreement is found also in the values of the first maximum in the curves with natural and artificial sap, summarized in the following table:

		Potential differences with	
		Sea water	<i>Valonia</i> sap
		mv.	mv.
Experiments with natural sap (12 measurements on 7 cells)	Extremes.....	4.1-13.0	42.4-74.9
	Mean.....	7.6	61.3
	Average deviation from mean..	2.2	6.5
Experiments with artificial sap (16 measurements on 9 cells)	Extremes.....	4.6-13.3	42.6-81.0
	Mean.....	8.2	63.0
	Average deviation from mean..	2.1	7.6

In some cases, later maxima in the P.D.-time curves were considerably higher than the first maxima reported in the above table. The highest P.D. observed was 100.5 mv. (with artificial sap).

Comparison of the values for P.D. with sap in this report and in earlier reports^{2,3} shows that our recent values are much higher than the values formerly reported as usual (25-35 mv.) and more like the value (82 mv.) formerly reported as unusually high. Since it is known that the K ÷ Na ratio in *Valonia* sap varies greatly among healthy cells depending on various factors, especially on the amount of illumination which the cells have received,⁶ it is not surprising that the P.D. produced by potassium-rich solutions should vary. The cells used in the earlier experiments had been kept in the laboratory, and hence had been much less strongly illuminated than the cells used in the recent experiments, which had been stored out of doors exposed to light from the sky, although shaded from direct sunlight. The effect of illumination on the P.D. requires further study.

toplasm. In a number of the time curves obtained in recent experiments, however, the P.D. fluctuated in a somewhat more complicated manner as shown in Fig. 2. Here we find the P.D. passing rapidly through two maxima in the first 15 to 20 minutes, then falling to a second minimum and rising slowly to a third maximum. It is interesting to see whether this more complicated behavior can be explained from the same simple assumptions.

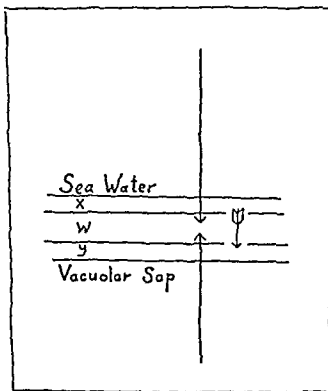


FIG. 3. Hypothetical diagram illustrating the theory of protoplasmic layers. The length of the arrows indicates the relative magnitudes of the P.D.'s assumed to exist across the inner and outer surface layers. The direction of the arrows is that in which positive current tends to flow. The resultant P.D. (the value observed) is shown by a feathered arrow.

The large P.D. which is observed with the system, sap/protoplasm/sap, led to the conclusion that the protoplasm itself is unsymmetrical; *i.e.*, that its external and internal surface layers are different. In their electrical behavior and in certain other respects¹⁰ these surface layers act like non-aqueous films immiscible with water. As a working hypothesis, the protoplasm is regarded (Fig. 3) as made up of an outer, non-aqueous layer, *X*, the aqueous main body of the protoplasm, *W*,

¹⁰ The nature of the protoplasmic surfaces is discussed by Osterhout, *Biol. Rev.*, 1931, 6, 397-9.

and an inner non-aqueous layer, Y , different from X . The observed p.d. is then the algebraic sum of the e.m.f. across X between the external solution and W , plus the e.m.f. across Y between W and the vacuolar sap. For the case of a cell immersed in sea water it is assumed that these e.m.f.'s have opposite signs, W being positive in an external circuit to both the vacuolar sap and the external sea water, and that both e.m.f.'s are large as compared with their algebraic sum. These assumptions are based on the changes in p.d. observed when a cell is killed by the addition of a toxic non-electrolyte to the external sea water under such conditions that X may be supposed to have been destroyed before enough of the toxic substance has diffused through W to produce much injury at Y .¹¹

In Fig. 4, $X-X$ is a hypothetical curve representing the sort of changes which we may expect in the p.d. across the outer surface of the protoplasm, X , when sap replaces sea water as the external solution, if we assume that the only change in the protoplasm is an increase in the concentration of KCl in the aqueous layer, W . (We need not consider here the mechanism by which K and Cl are transferred through the non-aqueous surface layers, X and Y , whether as ions, undissociated KCl, undissociated KOH and HCl, or in some other form.) The first rapid rise in p.d. caused by changing from sea water to sap is followed by a fall in p.d. as the concentration of KCl at the inner surface of X increases, approaching that at the external surface. Since we may suppose that the concentration of KCl in the main body of the protoplasm, W , is initially very low, the penetration of a small amount of KCl into W will at first cause a great decrease in the ratio $\frac{\text{KCl outside}}{\text{KCl in } W}$. As a result, we may expect that the p. d. will decrease at first at a correspondingly rapid rate, but that this rate will fall off as the concentration of KCl in W increases.

Meanwhile, KCl diffusing across W will reach the inner surface of the protoplasm and will produce a similar decrease in the p.d. there. As at the outer surface, this decrease will presumably be very rapid at first, but the rate will then fall off. (The concentration of KCl in W does not necessarily increase at a uniform rate: the entrance of KCl

¹¹ Unpublished results. For analogous experiments with *Nitella*, see Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927-28, 11, 673.

from the external solution may be partially offset by diffusion from *W* into the vacuole; furthermore, an increase in the concentration of salts in the protoplasm may alter its permeability.) The curve Y-Y

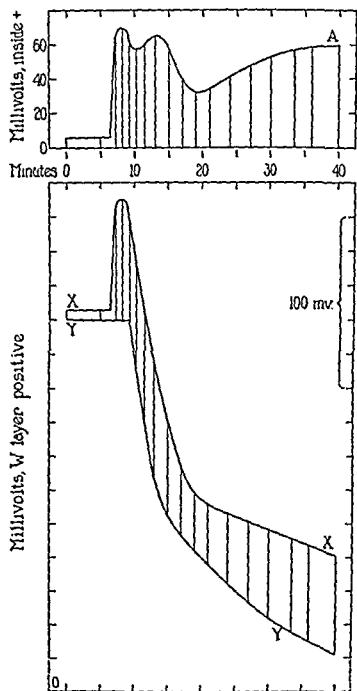


FIG. 4. Curves in which the observed P.D. across *Valonia* protoplasm in contact with *Valonia* sap (Curve A) is represented as the difference between oppositely directed E.M.F.'s at the outer (X-X) and inner protoplasmic surfaces (Y-Y). Differences between the ordinates of Curves X-X and Y-Y are equal to the corresponding ordinates of Curve A directly above. Curve A represents an actual experiment, Curves X-X and Y-Y are hypothetical.

in Fig. 4 shows these hypothetical changes in the P.D. across the inner surface layer of the protoplasm between *W* and the vacuolar sap. The P.D.'s plotted in X-X and Y-Y have opposite signs as in Fig. 3. The observed P.D. is then represented by differences between Curves X-X and Y-Y. This difference is represented in Curve A (above) the ordinates of which are equal to the difference between the corresponding ordinates of Curves X-X and Y-Y. Curve A, which obviously has the same general shape as the P.D.-time curves in Fig. 2, represents an actual measurement with artificial sap, the observed values being indicated by the ordinates which have been drawn.

It should be emphasized that while Curve A represents an actual experiment, Curves X-X and Y-Y are purely imaginary and may equally well be drawn in other ways. They are intended to show merely that in order to explain the fluctuations in P.D. with sap, we need not assume that the protoplasm suffers any further alteration than an increase in the concentration of KCl. If this explanation is correct, it is apparent from Fig. 4 that the values of the P.D. across the surface layers of *Valonia* protoplasm must be very large. The actual values are of course unknown; this is shown in Fig. 4 by interrupting the scale of ordinates toward the base by a dotted line, to indicate that below this point the ordinate extends for an indefinite distance.

SUMMARY

Evidence that the inner and outer protoplasmic surfaces in *Valonia* are unlike is found in the high P.D. across the protoplasm when the external solution has the same composition as the vacuolar sap. Earlier experiments with artificial sap have been repeated, using natural as well as artificial sap. Good agreement between the data with the natural and the artificial solution was found both in the magnitude of the P.D.'s observed and in the shape of the P.D.-time curves. The P.D.'s, however, were considerably higher than the values formerly reported as usual, while the cells proved much less liable to alteration produced by exposure to sap. It is suggested that the cells used in the recent experiments were in a more vigorous condition, perhaps as a result of exposure to stronger illumination.

The interpretation of the shape of the P.D.-time curves, proposed in an earlier report, and based on the theory of protoplasmic layers, is further discussed. It is assumed that the fluctuations in P.D. are due to an increase in the concentration of K in the main body of the protoplasm.

THE ACCUMULATION OF ELECTROLYTES

IV. INTERNAL VERSUS EXTERNAL CONCENTRATIONS OF POTASSIUM

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Although sea water contains a variety of cations the only one that accumulates in *Valonia macrophysa* is K^+ : this becomes about forty times as concentrated inside as outside. It is of considerable interest to ascertain how this process depends on the external concentration. This question is discussed in the present paper.

A previous investigation by S. C. Brooks¹ will be considered in connection with the discussion of our data.

EXPERIMENTAL

As pointed out in a previous paper² the $K \div Na$ ratio of cells growing in their natural state is not constant. Moreover the necessary handling and separation of the cells after collection, and the period of seasoning in the laboratory required for "healing," aggravate this difficulty. It is necessary among other things to control carefully the distribution of sizes of the cells taken for analysis, for it has been shown² that large cells from a lot of assorted sizes collected at the same time and treated in the same manner, may have a lower K and a higher Na content than small cells from the same lot. Doubtless the best procedure is to use cells alike in size and history, but this cannot always be done.

In our experiment the exposure to the modified sea waters was preceded by a period of exposure to running normal sea water containing $0.011 M K$ (as used throughout the experiment), to accustom the cells to a new environment before introducing other new variables. Before exposure and at intervals thereafter samples of sap from the cells were analyzed for hydrogen ion (pH), halide, Na , and K . At the same time the volumes of a group of cells set aside for the purpose were determined.

Three artificial sea waters, based on the directions of McClendon, Gault, and Mulholland³ but omitting minor constituents such as $LiCl$, sodium silicate, etc.,

¹ Brooks, S. C., *Protoplasma*, 1929, 8, 389.

² Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1930-31, 14, 301.

³ McClendon, J. F., Gault, C. C., and Mulholland, S., *Carnegie Institution of Washington, Pub. No. 251*, 1917.

were prepared containing respectively three times the normal amount of K, the normal amount of K, and no K. In all cases the total concentration of NaCl plus KCl was made the same. By mixing each of these sea waters with an equal volume of natural sea water there were obtained three sea waters, one containing twice the normal amount of K, another with the normal amount of K (the control), and one with one half the normal amount of K.

The cells were exposed as described in a previous paper² in glass troughs in large Pyrex glass tubes of 4 inches internal diameter, and 36 inches long. In order to avoid the slightest risk that the volume cells might become mixed with the cells for analysis, and so vitiate the volume measurements, they were placed in a 6 inch length of Pyrex tube 3 inches in diameter, closed at one end by cheese cloth secured by rubber bands, and at the other by a one-hole rubber stopper. The sea water from the reservoir was first conducted directly into the volume cell chamber by means of a glass tube inserted through the stopper, whence it escaped through the cheese cloth, passed over the other cells, and at length flowed out of the tube to the over-flow bottle. Thence it was pumped back to the reservoir bottle and recirculated. This continued throughout the experiment.

Each tube contained 450 cells plus 30 to 35 cells for volume measurement. Approximately 100 of these were used in preliminary analyses before the exposures to high and low potassium sea water began. The cells used were all collected at the same time and place, about one month before the start of the experiments. We were fortunate in having available a large number of cells of approximately the same size, 0.2 to 0.4 cc., so that cells much outside these limits were not used, which made it much less difficult to select representative lots for analysis.

The determination of volume was carried out in the apparatus shown in Fig. 1. It consisted of a U-tube having a wide arm *A* and a narrow arm *B*, bent nearly at right angles to *A*. When the levels in *A* and *B* were stationary a small addition caused a slight change of level in *A* and a much larger excursion in *B* which was read by means of a scale *C*, with a vernier reading to 0.1 mm. In order to measure the volume of an unknown object dropped into *A* the apparatus was calibrated by putting in an object of known volume and measuring the excursion in *B*.

To keep the angle of *B* constant with respect to the horizon during measuring and calibration, the U-tube was firmly fastened to a hardwood block *D* by "Picein" cement. Through the block was inserted a $\frac{1}{4}$ inch brass rod *E* which projected $\frac{1}{2}$ inch. A $\frac{1}{2}$ inch brass plate *F* was fastened firmly to a solid wall. This carried at one end a brass tube *G* of $\frac{1}{2}$ inch internal diameter, projecting at right angles from the face of the plate. This served as a bearing for *E*. The other end of the plate carried a $\frac{1}{2}$ inch brass bolt *H* tightly bolted through the plate and projecting about 1.5 inches. The free end of the block rested on this with the outer face of the wood block resting against the large head of the bolt. A wedge *J* inserted between the block and the brass plate prevented movement during the setting of the vernier.

Theoretically the angle assumed by *B* with the horizon should have been the same each time the apparatus was assembled. Under these conditions one cali-

bration would have been sufficient. Actually it was found simpler to permit small variations and to correct for these by calibration before each volume measurement. In no case did the correction exceed 1 per cent of the measured volume.

The pH values were determined by means of indicators and buffers. In order to decrease the salt error the buffers used for the sap were made up to approximately the same ionic strength as that of the sap by the addition of KCl: to those used for the sea water was added double strength artificial sea water. All the buffers were standardized by means of the hydrogen electrode. The indicator for the sap was adjusted to such a pH that its addition did not shift the equilibrium of the sap. This was tested in most cases by adding the indicator in two successive portions and determining the pH after each addition. If it did not change it was assumed that the indicator was isohydric with the sap, or at any rate that the sap was sufficiently buffered to overcome any effect of the indicator.

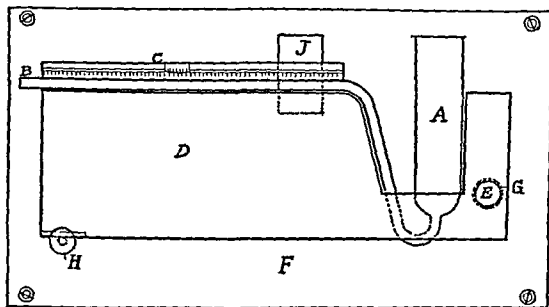


FIG. 1. Apparatus for measuring volume. Cells placed in *A* displace a column of water which is measured in *B*.

The indicator used with the sap was chlor phenol red, the pK of which according to Clark⁴ is 5.98. It was found in preliminary trials that the indicator when distinctly more acid or basic than this gave the same reading with the same sample of sap, *i.e.*, 5.95. Hence it was concluded that the sap is slightly buffered. Sea water is, of course, so well buffered that its pH value is not altered by the indicator.

To aid the eye in reading the pH to the nearest 0.05 pH unit a Hellige double wedge comparator was used. But since the calculation usually employed with this instrument takes no account of salt effects it was calibrated empirically by the use of the buffers described above.

⁴ Clark, W. M., The determination of hydrogen ions, Baltimore, The Williams & Wilkins Co., 3rd edition, 1928, 157.

The halide was determined by titration with silver nitrate using dichlorofluorescein as indicator as suggested by Kolthoff, Lauer, and Sunde.⁵

K and Na were determined together as the chloride and K as perchlorate. Thence the Na was calculated by difference. In some experiments the Na was determined on separate samples, as the sodium zinc uranyl acetate, as recommended by Barber and Kolthoff.⁶ It was found that the Na determined in this way agreed within less than 1 per cent with that obtained by difference.

The experiments were carried out in Bermuda in January and February. The exposure was made out of doors, but in such a way that the tubes were protected

TABLE I
Analytical Data for the Sap

K conc. in S. W.	Days	Volume	pH	Molecular concentrations				Ratio K/Na
				K	Na	K + Na	Halide	
0.006 M (low)	0	9.70	5.95	0.4751	0.1395	0.6146	0.6098	3.41
	3	9.77	6.05	0.4651	0.1374	0.6025	0.6003	3.39
	6	9.68	6.15	0.4602	0.1535	0.6137	0.5997	3.00
	10	9.67	6.05	0.4560	0.1549	0.6109	0.6003	2.94
	20	9.78	6.10	0.4433	0.1713	0.6146	0.5985	2.59
0.011 M (normal) Control	0	9.69	5.95	0.4751	0.1395	0.6146	0.6098	3.41
	3	9.81	6.05	0.4660	0.1446	0.6106	0.5967	3.22
	6	10.01	6.10	0.4679	0.1381	0.6060	0.5943	3.54
	10	10.27	5.95	0.4625	0.1443	0.6068	—	3.21
	15	10.50	5.90	0.4586	0.1443	0.6029	0.5967	3.18
	20	10.73	6.00	0.4531	0.1453	0.5984	0.5967	3.12
0.024 M (high)	0	9.65	5.95	0.4751	0.1395	0.6146	0.6098	3.41
	3	9.85	5.90	0.4823	0.1582	0.6405	0.6253	3.05
	6	10.07	6.00	0.4898	0.1460	0.6358	0.6253	3.35
	10	10.27	5.90	0.4869	0.1504	0.6373	—	3.23
	15	10.47	5.95	0.4915	0.1480	0.6395	0.6259	3.32
	20	10.57	6.15	0.4884	0.1446	0.6330	0.6253	3.38

from direct sunlight and to a great extent from the wind. The temperature varied from 16° to 20°C. In all cases the total mortality was less than 1 per cent.

The data are given in Tables I to III and are plotted in Figs. 2 to 5 (the curves being drawn free-hand, to give an approximate fit).

⁵ Kolthoff, I. M., Lauer, W. M., and Sunde, C. J., *J. Am. Chem. Soc.*, 1929, 51, 3273.

⁶ Barber, H. H., and Kolthoff, I. M., *J. Am. Chem. Soc.*, 1928, 50, 1625; 1929, 51, 3233.

They show that increasing K in the sea water from 0.011 M to 0.024 M causes an increase of K in the sap. A greater increase occurs when the

TABLE II
Analytical Data for the Sea Waters

Description	At the start			At the end		
	K	Halide	pH	K	Halide	pH
Low potassium sea water.....	0.006	0.5818	8.0	0.006	0.5848	8.0
Normal potassium sea water.....	0.011	0.5686	8.0	0.011	0.5704	8.0
High potassium sea water.....	0.024	0.5830	8.0	0.024	0.5877	8.0

TABLE III

K conc. in S. W.	Days	Gram molecules $\times 10^3$ = mols per liter \times volume in cc.			
		K	Na	Halide	K + Na
0.006 M (low)	0	4.608	1.354	5.915	5.962
	3	4.545	1.342	5.865	5.887
	6	4.445	1.486	5.805	5.931
	10	4.409	1.498	5.805	5.907
	20	4.331	1.674	5.921	6.005
0.011 M (normal) Control	0	4.608	1.354	5.915	5.962
	3	4.576	1.420	5.860	5.996
	6	4.689	1.384	5.955	6.073
	10	4.755	1.484	—	6.239
	15	4.820	1.517	6.272	6.337
0.024 M (high)	20	4.867	1.561	6.409	6.428
	0	4.608	1.354	5.915	5.962
	3	4.775	1.567	6.191	6.342
	6	4.958	1.478	6.329	6.436
	10	5.026	1.553	—	6.579
	15	5.173	1.558	6.587	6.731
	20	5.189	1.536	6.644	6.725

sea water contains 0.048 M K, as is clearly demonstrated by preliminary experiments carried out by L. L. Burgess.⁷

We also see an increase in the K \div Na ratio in the sap as compared

⁷ Owing to extreme fluctuations in the weather the controls in these experiments showed so much variation that we preferred to repeat the experiments under better conditions and use the preliminary experiments only for qualitative comparison.

with the control, and this is much more marked in the experiments of Burgess with 0.048 μ K in the sea water.

There is likewise an increase in the rate of penetration of K which becomes evident when we eliminate the disturbing effect of the penetration of water on the concentrations in the sap. In order to do this we plot a time curve showing the total amount of K entering the entire lot of cells during their growth. To obtain this we multiply the

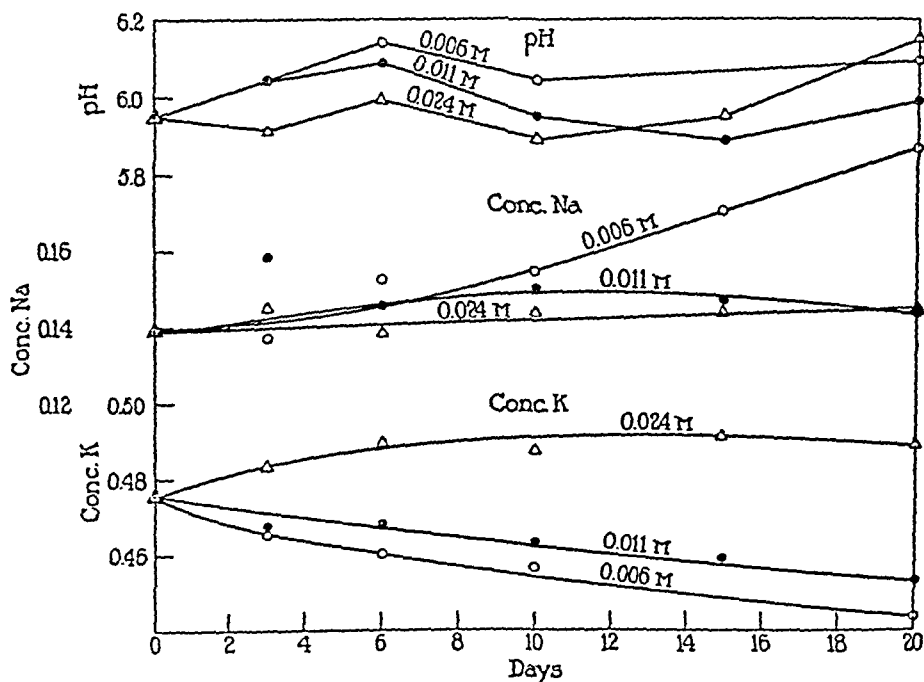


FIG. 2. Graphs showing changes in pH value and concentration of K and Na. Normal sea water with 0.011 μ K (\bullet); sea water with 0.006 μ K (\circ); sea water with 0.024 μ K (Δ).

concentration in the sap by the volume.⁸ This gives the curves labelled "mols K" in Fig. 4.

⁸ To facilitate comparison the volumes in sea water with 0.011 μ K were multiplied by 1.002 and those in sea water with 0.024 μ K by 1.005 which made them agree at the start with the volume in sea water with 0.006 μ K. It should be remembered that the rate of increase in volume of the cells used for analysis was not measured but inferred from the rate of increase of similar cells kept under the same conditions.

It will be observed that the concentration of K and the ratio $K \div Na$ fell off somewhat in the control (due presumably to conditions of illumination and temperature), but this was much more marked when the concentration of K in the sea water was lowered to 0.006 M (Figs. 2, 3, and 5).

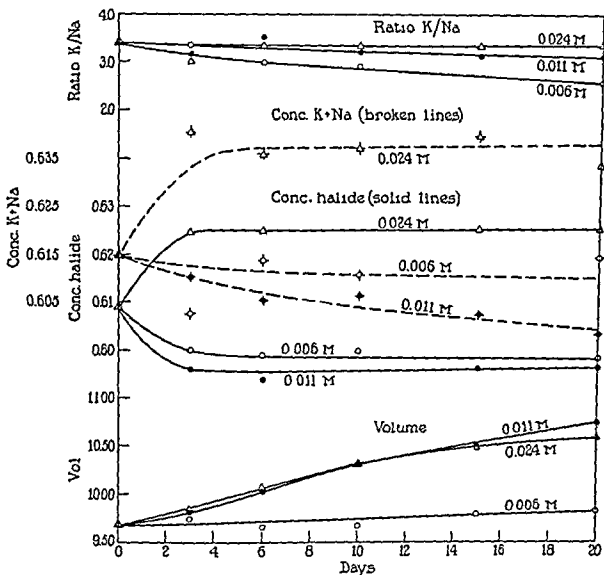


FIG. 3. Graphs showing changes in the ratio $K + Na$, in the concentration of total cations ($K + Na$), in the concentration of halide, and in volume. Normal sea water with 0.011 M K (\bullet) and (\blacklozenge); sea water with 0.006 M K (\circ) and (\blacklozenge); sea water with 0.024 M K (Δ) and (\blacklozenge).

We see an actual increase in the mols of K in the control and an actual decrease in sea water containing 0.006 M K (Fig. 4). This was also found in the preliminary experiments of Burgess with 0.006 M K in the sea water. He made analyses after 3, 7, 12, 18, 25,

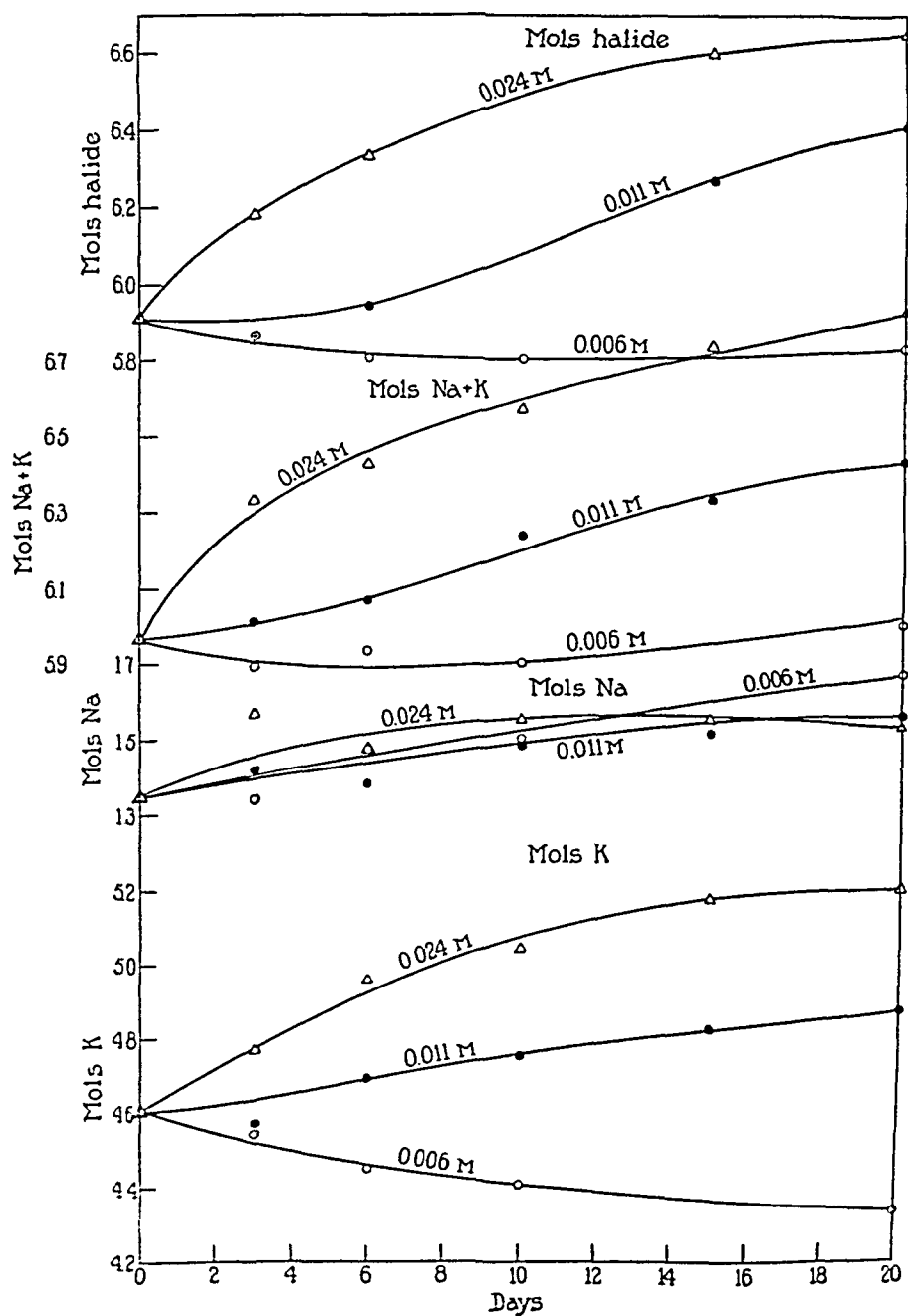


FIG. 4. Graphs showing changes in mols ($\times 10^3$) of halide, of $\text{Na}_2\text{S}_2\text{O}_8 + \text{K}$, of Na, and of K. Normal sea water with 0.011 M K (\bullet); sea water with 0.006 M K (\circ); sea water with 0.024 M K (Δ)

and 32 days, all of which showed smaller values for concentration of K, mols of K, and for $K \div Na$ than at the start, as well as smaller values than in the control.

We are therefore unable to agree with Brooks¹ that "The outstanding experimental fact . . . is that when living cells of *Valonia macrophysa*

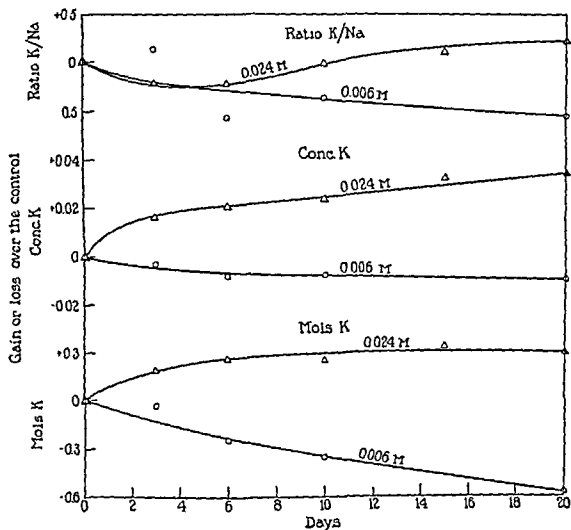


FIG. 5. Graphs showing gain or loss, as compared with the control, of the ratio $K \div Na$, of concentration of K, and of mols ($\times 10^3$) of K. Sea water with 0.006 M K (o); sea water with 0.024 M K (Δ).

are exposed to sea water so modified as to greatly decrease its potassium content, there occurs an increase of potassium concentration in the sap."

The decrease in mols of K in cells in sea water with 0.006 M K (seen in Fig. 4) means that K actually leaves the cell. This would of course occur if the cells were injured but there is no evidence that this

was the case. They appeared healthy in every way and the sap showed no more SO_4 than that of the control cells, nor was the mortality greater (it amounted to less than 1 per cent during the entire experiment). The growth was less than that of the controls, but this would be expected of uninjured cells under these conditions for reasons which will be discussed presently. It may be added that cells can be kept in ordinary sea water for long periods in the laboratory without growth and without showing any signs of injury.

It should be noted that the exit of K and cessation of growth are evident by the 3rd day. If they denote injury we should expect to see some signs of it during the following 17 days of the experiment, but this did not take place.

It may be added that all these statements apply equally well to the preliminary experiments of Burgess.⁹

Assuming therefore that these cells were not injured, how is the exit of K to be explained? According to the hypothesis developed in earlier papers¹⁰ the exit of K is to be expected¹¹ when $(\text{K}_i)(\text{OH}_i)$ is greater than $(\text{K}_o)(\text{OH}_o)$ (where the subscripts *i* and *o* denote activities inside and outside). This seems to be the case at 3 and at 6 days and would be so throughout the experiment if the pH value of the sap were in reality a little higher than our determinations make it out to be. This becomes evident from the following calculation. If we regard the activity coefficient of K^+ in the sap as identical with that of Cl^- and equal to that of K^+ in 0.611 M KCl, the activity coefficient¹² is 0.63.

The value 0.611 M is the average of the values for K + Na in Table I for sea water containing 0.006 M K. We neglect the effect of Ca in calculating the ionic strength of sap since we regard its concentration

⁹ At 32 days the volume was much nearer that of the control than in our experiments.

¹⁰ Osterhout, W. J. V., *Proc. Soc. Exp. Biol. and Med.*, 1928-29, 26, 192; *Biol. Rev.*, 1931, 6, 369. Cooper, W. C., Jr., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1930-31, 14, 117. Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1930-31, 14, 301.

¹¹ Such an exit has been observed under the influence of ammonia (Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1930-31, 14, 301).

¹² This value is obtained by interpolation from Harned, H. S., *J. Am. Chem. Soc.*, 1929, 51, 416.

in normal sap as less than 0.0017 M.¹³ Taking the ionic strength of the sea water to be 0.721 as calculated by Zscheile,¹⁴ we arrive at 0.62 as the value of the activity coefficient¹² of K in the sea water. Using these values and taking the pH value of the sea water as 8.0 (so that $\text{OH}_i = 10^{-6}$) we obtain the values given in Table IV.

It is evident that in some cases (e.g. at 3 and 6 days) the value of the product $(K_i)(\text{OH}_i)$ is greater than that of $(K_o)(\text{OH}_o)$; in others (e.g. at 10 and 20 days) it would be necessary to raise the pH value of sap only 0.07 pH to make $(K_i)(\text{OH}_i)$ greater than $(K_o)(\text{OH}_o)$ and this would be true even at the start if the pH value of the sap were 0.15 higher. If therefore our determinations of the pH value of the sap

TABLE IV

Values of the product $(K_i)(\text{OH}_i)$ when $(K_o)(\text{OH}_o) = 10^{-8.43}$ (i.e. with 0.006 M K in the sea water) and the activity coefficient of K in the sap is 0.63.

Time	pH of sap	OH_i	Conc. K in sap	$K_i =$ 0.63 (conc. K)	$(K_i)(\text{OH}_i)$
days					
0	5.95	$10^{-8.05}$	0.4751 = $10^{-0.323}$	$10^{-0.52}$	$10^{-8.57}$
3	6.15	$10^{-7.85}$	0.4651 = $10^{-0.333}$	$10^{-0.53}$	$10^{-8.38}$
6	6.15	$10^{-7.85}$	0.4602 = $10^{-0.337}$	$10^{-0.54}$	$10^{-8.39}$
10	6.05	$10^{-7.95}$	0.4560 = $10^{-0.341}$	$10^{-0.54}$	$10^{-8.49}$
20	6.10	$10^{-7.90}$	0.4433 = $10^{-0.353}$	$10^{-0.55}$	$10^{-8.45}$

were a little too low or those of the sea water a little too high, the exit of K in these cases would be explained and this might be the case owing to the uncertainty of the pH measurement.¹⁵

¹³ Cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1922-23, 5, 225.

¹⁴ Zscheile, F. P., Jr., *Protoplasma*, 1930, 11, 481. The activity coefficient calculated for the sap by Zscheile is in error since, owing to a typographical error the calcium in the analysis given by Osterhout (*J. Gen. Physiol.*, 1922-23, 5, 225) is ten times too large in the column where it is expressed as parts per thousand, although in the column where it is expressed as a molecular proportion the correct value is given. The use of the incorrect analysis makes the calculation of the ionic strength of the sap appreciably too high. We have used the more recent results of Harned¹² in place of those of Lewis and Randall.

¹⁵ By the uncertainty of the pH measurement, in this case we mean the uncertainty which attaches to all pH measurements due to the difficulty, not yet solved, of determining the activity of the hydrogen ion in the standard solutions on which

It follows from what has been said that a decrease of the pH value of the sap might cause the internal product (K_i) (OH_i) to fall below the outer and in that case K should enter. If this occurred in the experiments of Brooks it might explain his results.

It may be added that in our experiments with normal sea water and with increased K the external product was always larger and K always entered.

The exit of K would lower the osmotic pressure and cause a loss of volume were it not for the fact that Na continues to enter. This entrance would be expected since the product (Na_o) (OH_o) is more than 100 times as great as that of (Na_i) (OH_i). But the permeability¹⁰ to NaOH is so much less than to KOH that the entrance of NaOH just suffices to maintain osmotic pressure and the volume of the cell.

It might be suggested that during the exit of K there is an ionic exchange of K for Na, *i.e.*, that K^+ and Na^+ pass as such through the non-aqueous layers of the protoplasm. This would keep the total mols of Na + K constant throughout the experiment, as appears to be the case. But the facts can also be explained by saying that as there is practically no change in the external concentration of Na it continues to enter at the same rate as before, which compensates for the loss of K and that K and Na pass chiefly in molecular form through the non-aqueous layers, as elsewhere assumed.¹⁰

As already noted, an increase in the external concentration of K causes an increase in the ratio $K \div Na$, as compared with the control,¹⁸ and *vice versa* as is to be expected. Probably the same factors (presumably illumination and temperature) which caused a falling off in the control prevented any absolute rise in the cells in sea water with increased K.

We suppose that¹⁰ when KOH enters it combines in the sap with a weak acid HA and forms KA , thus raising the osmotic pressure, and by exchange with the sea water KA becomes KCl .

the whole hydrogen ion system is based, especially at high ionic strength and at high and low pH values. (Cf. Clark, W. M., Determination of hydrogen ions, Baltimore, The Williams and Wilkins Co., 1928, 3rd edition; MacInnes, D. A., and Belcher, D., *J. Am. Chem. Soc.*, 1931, 53, 3319.)

¹⁶ Except at the first determination when the mechanical disturbance connected with handling the cells at the start is probably responsible.

The increase in osmotic pressure would cause water to enter, provided the cellulose wall expanded; if it failed to do so (or to keep pace in its expansion with the increase in osmotic pressure) there would be an increase in the concentration of electrolyte in the sap.

Under normal conditions the cell wall appears to expand as fast as the osmotic pressure increases, so that the composition of the sap remains approximately constant, just as it does in certain models where there is no restraining membrane.¹⁷ But when we increase the amount of the more rapidly penetrating substance so that the osmotic pressure increases at an abnormal rate this no longer applies.

In the present case we see that the increase in external K produces an increase in the concentration of electrolyte (*i.e.* of K + Na, as well as halide) and an increase in the ratio $K \div Na$, but no increase in volume as compared with the control. We should therefore say that the cell wall did not expand at an increased rate so as to keep pace with the more rapid increase of osmotic pressure caused by the more rapid penetration of K. But when the external K was increased still more (from 0.024 M to 0.048 M), as in the preliminary experiments of Burgess, there was an increase in rate of growth as well as in the concentration of electrolyte in the sap. This is true in still greater degree when a more rapidly penetrating substance, such as ammonia, is added.²

When the external K was decreased from 0.011 M to 0.006 M, K came out about as fast as Na went in, so that the mols of Na + K remained nearly constant. As the osmotic pressure inside was higher than outside¹⁸ at the start the cellulose wall continued to expand for a time even after K ceased to enter, causing a slight increase in volume and a slight decrease in the concentration of electrolyte in the sap. At the start the control showed about the same decrease.¹⁹

¹⁷ Osterhout, W. J. V., and Stanley, W. M., *Proc. Soc. Exp. Biol. and Med.*, 1931-32, 29, 577.

¹⁸ The concentration of halide inside was 0.6098 M and outside 0.58 M.

¹⁹ According to the figures in Table I the increase in volume in sea water with 0.006 M KCl was about 1 per cent and the decrease in halide about 1.6 per cent. In other words they agree within the limits of experimental error. The decrease of halide in the control was about 2 per cent and the increase in volume about 1.2 per cent.

This may possibly be mechanical disturbance involved in handling the cells at the start.

After the first determination (at 3 days) the volume of the cells in sea water with 0.006 M K was approximately constant while that of the controls increased.

It will be noticed that the concentrations of total cations ($K + Na$) do not always agree with those of halide. This may suggest that varying amounts of HCO_3 are present, as would be expected if K and Na penetrated chiefly as KOH and NaOH but not if they penetrated as halides.

It would seem that in general the behavior of the cells is in accord with the hypothesis previously developed.¹⁰ As K penetrates more rapidly than Na its effect on growth and osmotic pressure predominates. Increasing external K to 0.048 M and decreasing external Na by 0.036 M, as in the experiments of Burgess, caused a decided increase in growth. When external K was increased only to 0.024 M there was no increase in growth but the osmotic pressure increased (as shown by the figures for halide) and presumably growth would have increased had the cell wall expanded to keep pace with the increase of osmotic pressure. Diminishing external K, and increasing external Na (by 0.006 M) diminishes growth. And, as would be expected, when we diminish external K to the point where $(K_o)(OH_i)$ is greater than $(K_i)(OH_o)$ the results indicate that K begins to come out but Na continues to go in, since $(Na_o)(OH_o)$ is greater than $(Na_i)(OH_i)$.

SUMMARY

Lowering the potassium in the sea water from 0.011 M to 0.006 M caused an exit of potassium from cells of *Valonia macrophysa*. Sodium continued to penetrate and the ratio $K \div Na$ fell off. The cells ceased to grow but there was no evidence of injury.

Increasing the external potassium brought about an increase of the internal concentration of potassium, of halide, of total cations, and of the ratio $K \div Na$ inside.

These phenomena are to be expected on theoretical grounds.

THE ADSORPTION OF GELATIN BY COLLODION MEMBRANES

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The fact that a collodion membrane in contact with a protein solution becomes coated with a film of protein was observed by Loeb (1). Some of the conditions governing the extent of this film formation were studied by Hitchcock (2). He found that the amount of adherent protein increased with the permeability of the membrane, and that, for membranes of any fixed permeability, the amount adsorbed reached a constant limiting value, independent of the protein concentration, in solutions of fairly high concentration. Hitchcock also studied the effect of the pH of the protein solutions on the amounts adsorbed by the membranes, finding the maximum adsorption at the isoelectric point of the protein. He found that this effect of pH could be abolished by the presence of a neutral salt. These effects, however, he studied at only one protein concentration.

The object of the present experiments was to find out, by varying the protein concentration in solutions of several different pH values, whether the *maximum* amount adsorbed varied with the pH, in order to obtain further information as to the state of the protein in solution and to test possible explanations of the pH effect.

Experimental Methods

Preparation of Membranes.—Membranes of a fairly high permeability were prepared by a method similar to that of Nelson and Morgan (3). A collodion solution containing about 4 gm. of solid per 100 cc. (Merck, U.S.P.) was diluted with an equal volume of a mixture of ordinary ether ("for anesthesia") and alcohol (95 per cent), the proportions being 3 volumes of ether to 1 of alcohol, which are

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about those in the original collodion solution. 10 cc. of this 2 per cent collodion were delivered from a pipette on to a circular glass plate, 10.1 cm. in diameter, floating on mercury. When the solvents had evaporated enough so that the collodion had set to a fairly rigid gel (usually after 9 or 10 minutes), the glass plate was placed on a balance and the solvents were allowed to evaporate further at room temperature until a predetermined weight was reached. The plate was then submerged in distilled water, and after the membrane had become loosened from the glass it was placed in fresh water and left overnight before its permeability was determined. From each of these membranes four disks were cut by a steel die 3.81 cm. in diameter, one disk being used to determine permeability and dry weight, and the remainder for the adsorption experiments. The permeability was determined, in arbitrary units, by noting the time required to force a given volume of water, under a known pressure, through a definite area of the membrane. The rate of flow did not become constant until a membrane had been under pressure for about 10 minutes, and in this time the membrane had become somewhat stretched, although a wire gauze support was used. Accordingly the figures obtained are not believed to represent absolutely the permeability of the unstretched membrane, but are used merely for comparative purposes. Membranes whose permeability did not agree to within 3 per cent were discarded; it was found that variations within this limit did not appreciably influence the amount of protein adsorbed. The dry weight of a disk was obtained after drying in an oven at 105°C. for 1½ hours. These weights were constant to 0.5 mg., their average being about 32.2 mg.

This method of preparing membranes was found to have the disadvantage that the permeability of the membranes so obtained is affected by the weather. To obtain membranes of lower permeability, another method was used. Following a suggestion of Schoep (4), small amounts of glycerine (about 1 per cent) were added to the diluted collodion. The collodion was poured, according to Bigelow (5), directly on a large surface of mercury, 120 cc. being used to cover a surface about 16 inches in diameter. The solvents were allowed to evaporate under a cover of cheese-cloth until the presence of alcohol and ether could no longer be detected, after which the membranes were treated with water as before. In this method the permeability is regulated only by the amount of the non-volatile glycerine present and the thickness of the membrane. Different portions of a single membrane made in this way were found to be uniform as to thickness and permeability, but the permeability of different whole membranes was not very reproducible. The large size of the membranes, however, made it possible to cut enough disks from each to run a complete adsorption experiment. Disks obtained from a single membrane were constant in dry weight to within 0.5 mg., but disks from different membranes varied somewhat, the range of dry weights being from 24.5 to 26 mg.

Preparation of Gelatin Solutions.—The gelatin used was the Eastman Kodak Company's "de-ashed" preparation, which is purified by them according to Northrop and Kunitz (6). Solutions were prepared by dissolving approximately the desired amount of air-dry gelatin in water or in dilute hydrochloric acid or

sodium hydroxide solution, according to the pH desired. Aliquot portions of these stock solutions were then diluted with varying amounts of solvent to give series of solutions of varying gelatin concentration. For solutions at the isoelectric point the dilutions were made with distilled water. For solutions on the acid side, the pH of the stock solution was determined by the hydrogen electrode (at 30°C.) and the aliquot parts were then diluted with hydrochloric acid of the same pH. For solutions on the alkaline side, pH 6 to 7, the dilutions were made with distilled water, as this did not change the pH enough to affect the adsorption.

The concentrations of gelatin in these solutions were determined by evaporating samples of known volume and drying to approximately constant weight at 105°C., the period of drying being not less than 18 hours.

Adsorption Experiments.—The method used was that described by Hitchcock (2). In each case three disks of membrane, 3.81 cm. in diameter, were agitated for 16 to 18 hours with 25 cc. of gelatin solution in tightly stoppered Pyrex glass tubes submerged in a water bath at 37°C. The disks were washed 3 times at 37° by stirring them for a few minutes in about 300 cc. of distilled water or dilute acid, according to the pH of the gelatin solutions, and they were then dried for 2 hours at 105°C. After the 1st hour at 105° the loss in weight was so slight as to be negligible. Similar determinations were made in each case with disks of the same membrane which had not been treated with protein, and the weights of adsorbed gelatin were obtained by difference. It had been shown by ash determinations (2) that the same method is applicable to the case of gelatin solutions containing salt as well as acid or alkali. In experiments in which sodium chloride was used, weighed amounts of the dry salt (Baker's Analyzed) were added to the aliquot parts of gelatin solution before the final dilutions were made, so that after dilution all the solutions in a series had the same concentration of salt.

EXPERIMENTAL DATA

The results of the experiments are presented graphically. The ordinates of each curve are the weights of dry gelatin, in milligrams, adsorbed from 25 cc. of solution by three disks of membrane, and the abscissae are the weights, in grams, of gelatin remaining in the 25 cc. of solution at equilibrium. On each chart is recorded also the pH value of the solution and the relative permeability of the membrane. This quantity, designated by m , is the product of the pressure used in the permeability determination and the time required for this pressure to force a fixed volume of water through a given area of membrane. With this notation membranes of high permeability have low values of m , and *vice versa*. The abbreviation I.E.P. on the charts means isoelectric point.

Fig. 1 gives the results obtained with membranes of rather high

permeability, prepared by the first method. Curve 1, representing experiments with solutions at or near the pH of the isoelectric point, shows that a limiting or saturation value was attained with about 6 per cent gelatin (1.5 gm. in 25 cc.). At lower pH values, 4.3 and 3.8, Curves 2 and 3 show that much higher gelatin concentrations were required to produce maximum adsorption. At pH 4.3 (Curve 2) the maximum was reached in about 10 per cent gelatin (2.5 gm. in 25 cc.), while at pH 3.8 (Curve 3) the curve was still ascending at a concen-

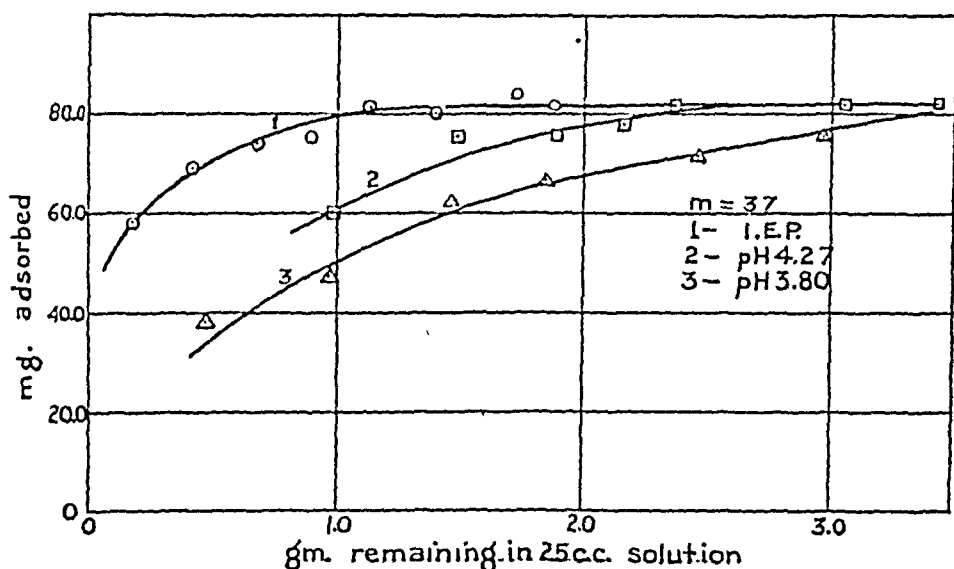


FIG. 1. Adsorption of gelatin by membranes of high permeability. Isoelectric gelatin and acid gelatin.

tration of 12 per cent. It was not practicable to work with solutions more concentrated than 12 or 14 per cent on account of their high viscosity. The three curves of Fig. 1 show that the maximum amount of gelatin which these permeable membranes could adsorb was not influenced by acidity, but that the pH did affect the value of the gelatin concentration required to produce this maximum adsorption.

Fig. 2 shows results obtained with membranes of much lower permeability, prepared by the second method. In this case, in agreement with Hitchcock (2), it was found that maximum adsorption was attained at relatively low concentrations of gelatin. Here, however, in contrast to the results obtained with the permeable membranes, it is

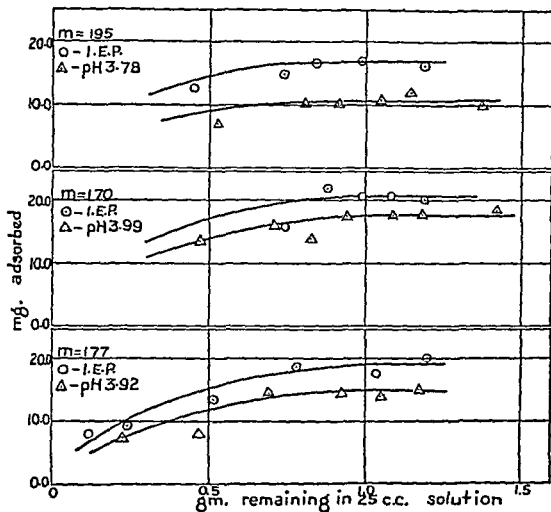


FIG. 2. Adsorption of gelatin by membranes of low permeability. Isoelectric gelatin and acid gelatin.

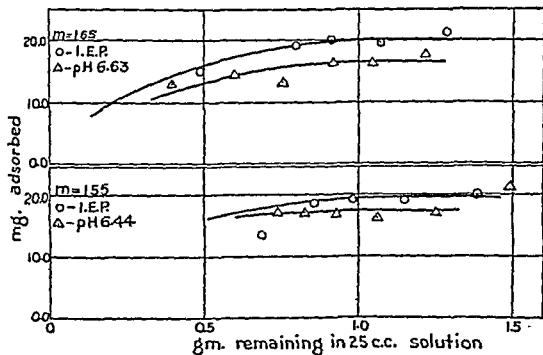


FIG. 3. Adsorption of gelatin by membranes of low permeability. Isoelectric gelatin and acid gelatin.

to be noted that in each case the maximum amount of gelatin adsorbed from solutions of lower pH, 3.8 to 4.0, was definitely less than that adsorbed from solutions near the isoelectric point (about pH 4.8).

Fig. 3 shows a similar effect on the alkaline side, pH 6.4 to 6.6. Here again, with membranes of low permeability, the maximum amount of gelatin adsorbed was less when the pH was not that of the isoelectric point.

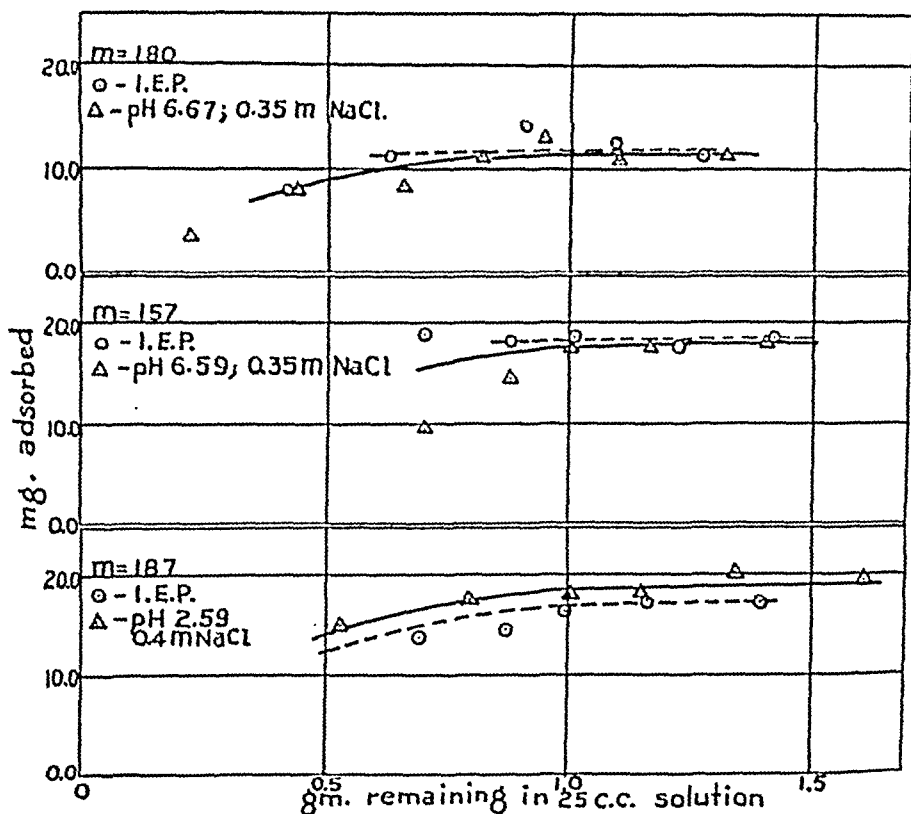


FIG. 4. Adsorption of gelatin by membranes of low permeability. Isoelectric gelatin without salt, and acid and alkaline gelatin with salt.

Fig. 4 shows that this effect of pH on the value of the maximum amount of adsorbed gelatin was practically obliterated by the presence of sodium chloride at concentrations of 0.35 or 0.4 molar.

DISCUSSION OF RESULTS

The explanation tentatively advanced by Hitchcock (2) for the effect of pH on the adsorption of gelatin by collodion membranes was

based on Loeb's (7) theory of the mechanism of the effect of electrolytes on the viscosity of gelatin solutions. According to this theory gelatin exists in solution as submicroscopic particles capable of swelling, the swelling being governed by the Donnan equilibrium. On this basis the volume of the particles should be least at the isoelectric point, and greater at pH values on either side of that point. Accordingly it should be possible for more particles to come into contact with a given surface of membrane if the solution is at the isoelectric point. On either side of this point, if the particles are larger, fewer particles can come into contact with the membrane surface, and hence the amounts adsorbed should be less.

The results in Fig. 1 appear at first glance to contradict this explanation, since the curves obtained at different pH values are evidently tending towards the same maximum value for the amount adsorbed. However, it was noted by Kunitz (8) that the effect of pH on the viscosity of gelatin solutions disappeared at high gelatin concentrations, in the vicinity of 10 per cent. This must mean that in such solutions variations from the pH of the isoelectric point do not increase the swelling of the particles. Fig. 1 shows that the effect of pH on the amounts adsorbed did not disappear until the concentrations of gelatin were 10 per cent or higher. Hence the identity of the maximum heights is not wholly inconsistent with Hitchcock's explanation on the basis of Loeb's theory if the additional facts obtained by Kunitz are considered. It is possible, however, that the data in Fig. 1 may mean that some factor other than the size of particles is effective in governing the amounts adsorbed. The difference in height between Curves 1 and 3 at the point corresponding to 10 per cent gelatin (2.5 gm. in 25 cc.) seems to be well outside the experimental error, yet according to Kunitz the effect of pH on viscosity, and hence on particle size, is negligible in solutions of this concentration. The amount adsorbed may be governed in part by a preferential adsorption of uncharged or isoelectric protein particles, as well as by the size of the particles.

In the remaining experiments, where membranes of lower permeability were used, the maximum of adsorption was attained at lower gelatin concentrations, in the vicinity of 4 per cent or less. In such solutions, according to Kunitz, distinct variations in viscosity with pH

are perceptible. Hence in these solutions variations in pH should affect the volume of the particles, so that the lowering of the maximum amount adsorbed as the pH was removed from that of isoelectric point is still explicable on the basis of Loeb's theory.

The effect of salt, illustrated in Fig. 4, is also in agreement with Loeb's theory. He found that the addition of salt to gelatin solutions containing acid or alkali depressed the viscosity just as it did the values of properties depending on the Donnan equilibrium. Accordingly he believed that the salt inhibited the swelling of submicroscopic particles of gelatin in solution by its depressing effect on the unequal distribution of diffusible ions. If this theory is adopted, it follows that a greater number of such particles should be able to reach the membrane in the presence of salt than in its absence, in solutions of pH different from that of the isoelectric point. Therefore the results shown in Fig. 4 may be explained on the basis of Loeb's theory.

The experiments of Hitchcock with egg albumin, in which he found an effect of pH on the amounts adsorbed quite similar to that observed in the case of gelatin, have not yet been explained. Because of the very slight effect of pH on the viscosity of egg albumin solutions, Loeb (7) believed that solutions of this protein did not contain particles capable of swelling, but only molecules and ions in true solution. It is hoped that a future study of adsorption from solutions of egg albumin, along the lines of the present experiments with gelatin, may shed some light on the differences in the nature of the particles of these two proteins in solution.

SUMMARY

An experimental study has been made of the adsorption of gelatin from solution at 37°C. by collodion membranes. In the case of membranes of high permeability, very high concentrations of gelatin were required to produce maximum adsorption, and the maximum amounts adsorbed were independent of the pH values of the solutions over the range 3.8 to 4.8. With membranes of low permeability, maximum adsorption was reached at lower gelatin concentrations, and the maximum amounts adsorbed varied with the pH, being lower on either side of the isoelectric point, over the range 3.8 to 6.6. The addition of salt in such experiments raised the maximum amount adsorbed

to a value equal to that obtained with solutions at the isoelectric point in the absence of salt.

These experiments can be explained by, and seem to lend support to, the theory proposed by Loeb and further developed by Kunitz concerning the effects of pH and salt on the size of gelatin particles in solution.

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ON THE RESPIRATORY QUOTIENT OF LUPINUS ALBUS AS A FUNCTION OF TEMPERATURE

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I

Previous studies on the temperature characteristics for the respiration of the germinating seeds of *Lupinus albus* have shown (Tang, 1930-1931, 1931-1932, *a*) the values of μ 's for consumption of oxygen and for production of CO_2 to be different. It was inferred that the respiratory quotient of the seeds must therefore be a function of temperature. The object of the present paper is to examine this point.

II

The Warburg microrespirometer technic (Warburg, 1926) was used in this series of experiments. The customary conical vessel was replaced with a cylindrical one of special design (Tang, 1931-1932, *b*). It is attached to the manometer from the side instead of the top which has a removable stopper bearing a glass cross on which the seed is fastened with a sulfur-free rubber band. 0.2 cc. of 2 N KOH is placed in the bottom of the vessel and 0.3 cc. of a 2.5 N HCl in the side arm of the ordinary type. The capacity of the vessel is about 15 cc. In practice, a set of seven respirometers was used: one for thermobarometer; three, containing only KOH, were used as adaptation chambers in which the seeds were allowed to remain at the desired temperature prior to the observation. After this period of adaptation, the seeds, which are attached to the crosses on the interchangeable stoppers, are removed to the other three vessels containing KOH and HCl. In this way the CO_2 formed during the period of adaptation was not absorbed by the KOH in the respirometer. Exactly 5 minutes after the seeds were placed in the respiring chambers the stop-cocks are closed and measurements begun. At the end of 2 hours, the oxygen consumed is measured by the negative pressure on the manometer. To this is added the amount of oxygen consumed during the first 5 minutes, by extrapolation. The amount of CO_2 produced is obtained by tipping the HCl in the side arm into the vessel proper and observing the positive pressure. From this amount must be subtracted the amount of CO_2 originally present in the

KOH solution, which is obtained by tipping the HCl in the side arm of the thermobarometer which contains amounts of solutions identical with those in the respirometer. The manometers are shaken fairly vigorously (*ca.* 60 complete oscillations per minute with a throw of 8 cm.) for 5 minutes after the tipping of the acid. This was found to be sufficient to liberate all the CO₂ from the alkali, without the manometric reading being altered by any gas exchange during that time.

The tipping of acid on the respiring material in order to obtain the "pre-formed" CO₂ as in the animal tissue suspended in aqueous solutions (Meyerhof, 1930; Gerard, 1927; Dickens and Šimer, 1930) was deliberately avoided, since preliminary experiments showed that not only was there no CO₂ given off when the seeds were so treated, but that the manometer actually showed a slight negative pressure which is not prolonged for more than a few minutes and is decidedly different from the phenomenon observed by Amberson, Armstrong, and Root (1931).

The pressure changes, multiplied by the suitable vessel constants, give the volumes of gas exchanged. The constants are given by the equation:

$$K = \frac{V_s \frac{273}{T} + V_f \alpha}{10,000}$$

in which V_s is the volume of the vessel minus those of the respiring material and liquid (V_f). T is the temperature of the experiment in Kelvin units, α the Bunsen coefficient of solubility of O₂ or CO₂ in water at T , and 10,000 is the atmospheric pressure in terms of Brodie solution (Warburg, 1926). The volumes of the vessels were calibrated by any convenient method (Oppenheimer, 1928; Warburg, 1926), and the volume of the seed was obtained by immersing it in water in a graduated centrifuge tube. Since the solubility of gases in water at different pH is not exactly known, V_f was made so small that the term $V_f \alpha$ vanished even if the Bunsen coefficient of solubility were taken, rendering incidentally the K for both CO₂ and O₂ identical.

After the necessary data were obtained, the temperature of the thermostat, which is maintained constant to 0.01°, is changed. The seeds are placed in the three original chambers for thermal adaptation while the three others, together with the thermobarometer, are removed for washing, cleaning, and refilling with new solutions. They are then returned to the thermostat, and after thermal equilibrium is attained, are used for the next experiment.

The seeds are the same as those used in the previous work (Tang, 1930-1931) and are treated in exactly the same way. The experiments were performed in darkness; the manometers were not shaken during the actual experiment.

III

For production of CO₂ by *Lupinus albus*, the value of μ was found to be $24,000 \pm$ calories, and for consumption of oxygen $16,600 \pm$

calories, below the critical temperature of about 19.5° . According to the Arrhenius equation, for CO_2 production we have:

$$\log K_2^{\text{CO}_2} - \log K_1^{\text{CO}_2} = \frac{\mu^{\text{CO}_2}}{R} \left(\frac{1}{T_1} - \frac{1}{T_2} \right) \quad (1).$$

Similarly, for oxygen consumption,

$$\log K_2^{\text{O}_2} - \log K_1^{\text{O}_2} = \frac{\mu^{\text{O}_2}}{R} \left(\frac{1}{T_1} - \frac{1}{T_2} \right) \quad (2).$$

Subtracting (2) from (1), we have

$$\log \frac{K_2^{\text{CO}_2}}{K_2^{\text{O}_2}} - \log \frac{K_1^{\text{CO}_2}}{K_1^{\text{O}_2}} = \frac{\mu^{\text{CO}_2} - \mu^{\text{O}_2}}{R} \left(\frac{1}{T_1} - \frac{1}{T_2} \right) \quad (3).$$

If K is measured in terms of volume (or units proportional thereto) per unit time, (3) becomes

$$\log RQ_{T_2} - \log RQ_{T_1} = \frac{\mu^{\text{CO}_2} - \mu^{\text{O}_2}}{R} \left(\frac{1}{T_1} - \frac{1}{T_2} \right)$$

Rearranging,

$$\log \frac{RQ_{T_2}}{RQ_{T_1}} = \frac{\mu^{\text{CO}_2} - \mu^{\text{O}_2}}{R} \left(\frac{1}{T_1} - \frac{1}{T_2} \right) \quad (4).$$

This is the general expression relating the respiratory quotient (RQ) to temperature and to the difference in the values of μ 's. It will be noted that it is the *difference* of the μ 's that is concerned here and not their absolute values. Substituting for μ_{CO_2} and μ_{O_2} the values 24,000 and 16,600 respectively, taking $R = 2$, T_2 as 291, and T_1 as 281, and converting to Briggsian logarithms,

$$\log_{10} \frac{RQ_{291}}{RQ_{281}} = 0.196 \quad (5)$$

and

$$\frac{RQ_{291}}{RQ_{281}} = 1.57 \quad (6)$$

Allowing a ± 5 per cent variation in the values of μ 's, as might be expected from the fluctuations of Q_{O_2} and Q_{CO_2} at constant tempera-

ture (*cf.* Tang, 1930-31, *a, b*), and taking the differences of the extreme values, the ratio in (6) should fluctuate between the limits 1.39 and 1.78. Table I gives the data obtained experimentally, using seeds from the same lot, with the same germination treatment as previously described (Tang, 1931-1932, *a*).

The mean of the five ratios of $R.Q._{221}/R.Q._{231}$ is 1.51 with extreme values of 1.35 and 1.71, which are in good agreement with those demanded in equation (6). The values of μ 's for both CO_2 production and oxygen consumption agree satisfactorily with those obtained previously. These values were obtained regardless of whether the

TABLE I
Respiratory Quotients of Germinating Seeds of Lupinus albus at 18° and 8°C., and the Calculated Value of μ

Exp. No.	1		2		3		4		5		
Temperature	15°	8°	15°	8°	15°	8°	15°	8°	15°	8°	Average
Q_{CO_2} , c. mm./seed/ hr.	78.0	20.5	76.0	18.0	61.0	15.0	39.5	8.5	36.0	9.0	
Q_{O_2} , c. mm./seed/ hr.	88.0	31.0	93.0	32.5	77.0	27.0	55.0	20.0	55.0	22.0	
R.Q.	0.89	0.66	0.82	0.55	0.79	0.56	0.72	0.42	0.65	0.40	
R.Q. 18°/R.Q. 8° ..	1.35		1.49		1.41		1.71		1.59		1.51
μ_{CO_2}	22,000		23,500		23,000		25,500		23,000		23,300
μ_{O_2}	17,100		17,200		17,200		16,600		15,100		16,400

seeds were subjected to the higher temperature first and then to the lower or *vice versa*, provided adequate time was given for thermal adaptations which in this case takes at least 2 hours. Table I shows also the variability of Q_{O_2} , Q_{CO_2} , as well as of the R.Q.'s, among the individual seeds. The uniformity of the Q 's is not materially improved when expressed in terms of fresh or of dry weights, or in terms of volume or surface of seed.

IV

The results of this series of experiments, together with those already published (Tang, 1931-1932, *a, b*), clearly bear out two interesting

points; *viz.*, the temperature characteristic for the production of CO_2 by *Lupinus albus* is different from that for the consumption of O_2 , and the respiratory quotient accordingly changes with temperature.

That the difference in the temperature characteristics for the two phases of respiration is neither an accidental occurrence nor a technical artefact is made evident by the fact that such a difference was not observed in *Zea* (Tang, 1931-1932, *b*), and also by the fact that it occurs in *Lupinus albus* regardless of the technic used.

Granting that the difference in the temperature characteristics is a real one, we are confronted by three possible interpretations of this phenomenon. The first and obvious one is that suggested in an earlier paper (Tang, 1931-1932, *b*), namely that the mechanisms governing the production of CO_2 and the consumption of oxygen may be different although acting simultaneously. This is not at all improbable, especially in the light of the modern conception of the relationship between fermentation and respiration (*cf.* Kostychev, 1927, Chapter 3). Indeed, recent investigations by Blackman and Parija (1928), and those of Chivellard *et al.* (1931), with apples, potatoes, mosses, etc., at different oxygen tensions come to almost the same conclusion. However, these authors are of the opinion that at higher oxygen tensions respiration (oxidation) takes place exclusively.¹ Although their conclusions may be entirely sound, one hesitates to give much weight to experiments where such bulky materials as apples and potatoes are used, and where the experiments are carried out over too long a time.

A second interpretation of the difference in the temperature characteristics might be offered by assuming a difference in the respiratory activities of the cotyledons and of the plantule, as in the case of *Ricinius* seeds discussed by Murlin (1932) among others. If this were true, it is hardly conceivable that any regularity should occur in treating the respiration of the whole seed as a function of temperature, and it is certainly inconceivable that the ratio of the respiratory quotients should come out exactly as predicted by the use of equation (4) which is derived from the assumption that the difference in the temperature characteristics is a real one, due to specific differences in

¹ Blackman and Parija are of the opinion that glycolysis is the common process governing both oxidation and fermentation.

the mechanisms underlying the two processes. That the individual ratios in Table I deviate rather markedly from the average of 1.51 as pointed out above, is not a strong objection in this case. It must be remembered that in (6) the value of 1.57 is derived from a logarithmic value of 0.196 of (5). A small deviation in the latter caused by taking slightly different values of the μ 's, reflects a considerable variation in the former, as was anticipated. Moreover, the R.Q.'s are computed from observations at two temperatures only, and the temperature characteristics must be expected to show some variation when so obtained, as earlier pointed out, and the variations observed fall within expected limits.

A third explanation may be attempted by supposing an inadequacy of diffusion of oxygen into the seeds. If this were true, there would be created at least two zones in the seed—an outer one of oxidation, an inner one of anaerobic cleavage; the depth of the former depending on the rate of diffusion of oxygen into the seed. Had the coefficient of diffusion of oxygen into plant materials been known, as in the case of animal tissues (*cf.* Krogh, 1919; Tang and Gerard, 1932), the problem could have been dealt with by a simple calculation (*cf.* Warburg, 1926; Hill, 1928–1929). Unfortunately such data are unavailable, and we have to dismiss the question with the rather unsatisfactory statement that there was no significant rise in the rate of oxygen consumption of our seeds when they were subjected to an oxygen atmosphere as compared with their rate in air, indicating that the diffusion of oxygen is presumably not a limiting factor in this case. Even if diffusion were the limiting factor, the rate of production of CO_2 from the inner zone must be considerably higher than that of the outer (where both CO_2 production and O_2 consumption occur) in order to give an apparent and dominant temperature characteristic. Then, in all probability, the total amount of CO_2 produced per unit time by the seed as a whole will be in excess of the oxygen consumed. But in reality, the respiratory quotient, even at the higher temperature (18°), is never equal to unity, and is about 0.80.

Thus in view of absence of direct evidence against it, and also because of the close agreement of the ratios of the respiratory quotients at 18° and 8° with that demanded by equation (4), we may work on the hypothesis that in the seeds of *Lupinus albus* at least, the produc-

tion of CO_2 and the consumption of oxygen may be simultaneous and yet governed by two different mechanisms.

The second point of interest which can be deduced from the measurements, and is in reality a corollary to the preceding paragraph, is the change of respiratory quotient with temperature. In going over the literature on plant respiration, we find the question of the change of respiratory quotient with temperature an old one. While it is not the aim of this paper to give a comprehensive review of the literature, a brief summary may be made. Bonnier and Mangin (1884), Dehérain and Moisson (1874), and Aubert (1892), maintained that temperature changes do not affect the respiratory quotient; Pouriewitch (1905) later questioned their conclusions and showed in all the plant materials which he studied, including the seedlings of *Lupinus albus*, a change of the quotient with temperature. Recently Harrington (1923) also found a change in the respiratory quotient with temperature in his study of the respiration of apple seeds. These authors, however, attribute the change, if it does occur, to the change in the nature of the food materials being consumed. The experiments reported here give a possible explanation of the phenomenon other than that offered by Pouriewitch and Harrington (*cf.* also Kostychev, 1927, Chapter 1). If the mechanisms governing the production of CO_2 and the consumption of oxygen are different, and are affected differently by temperature, a change of the respiratory quotient with temperature is the necessary consequence. In such cases we ought to be able to predict the change not only qualitatively, but also quantitatively. This we have done. These two interpretations of the change of the respiratory quotient with temperature are not necessarily mutually exclusive. It is perfectly conceivable that in certain cases the kinds of food material may be the cause for the change, while in others the nature of the reaction mechanisms. The two may or may not be interdependent.

The writer is aware that the respiratory quotient has been thoroughly investigated in animals, especially in man, and he does not doubt the usefulness of the respiratory quotient in clinical investigations where it is treated solely as a function of food materials consumed—provided it be determined with due care (*cf.* Richardson, 1929; and Cathcart and Markowitz, 1927). Even in human respiration, the

quotient does change with the temperature of the body (Landis *et al.*, 1926); but there it is due principally to the change in the pH of the blood and to the difference in the rate of pumping out of the gases from the lung. The phenomenon is thus rendered complicated for analyses of the kind employed here.

SUMMARY

The temperature characteristics for the oxygen consumption and CO₂-production of the germinating seeds of *Lupinus albus* were previously found to be different. It was predicted qualitatively that the respiratory quotient of the seed should be a function of temperature. A quantitative treatment is presented here, relating the change of the respiratory quotient with temperature and the temperature characteristics. Experimental results agree satisfactorily with the calculated value.

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A RESPIROMETER VESSEL FOR STUDY OF METABOLISM OF SEEDS

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In a series of observations on the respiration of germinating seeds (Tang, 1930-1931; 1931-1932, *a, b*) use was made of the Warburg respirometer technic (Warburg, 1926). While this technic has enjoyed considerable popularity in other fields, its use in the investigation of the respiration of seeds has not been frequent. The only attempts known to the writer are those made by certain German workers (Ullrich and Ruhland, 1928; Stälfelt, cited by Pringsheim, 1931). This paper describes a modification of the Warburg vessel which is especially adapted to the study of the respiration of single seeds at rest or during geotropic excitation. For a comprehensive treatment of the history and principle of the technic, Krebs's article in Oppenheimer's handbook (1928) and Warburg's monograph (1926) may be consulted. An English translation of the latter is available (*cf.* also Burk and Milner, 1932). For other types of vessels using this general principle, the accounts by Harrington and Crocker (1923) and by Pringsheim (1931) may be useful.

The vessel is cylindrical in shape, 5 cm. tall and 2 cm. in diameter. It is attached to the manometer from the side instead of at the top as is the usual case. The reasons for this are: (1) the seeds can be suspended on a glass cross attached to the stopper capping the vessel, thus simplifying the problem of mounting; (2) it can be rotated, with the joint as an axis, to any desired angle in relation to gravity. Opposite to the joint, the vessel has a side arm of about 2 cc. capacity with a ground-in stopper. Into this side arm HCl, or any other liquid, may be placed, which can be tipped into the vessel proper if desired. The alkali for CO₂ absorption is placed in the bottom of the vessel proper, which has a capacity of about 15 cc. The vessel is open through the top and is closed with a ground-in glass stopper. In the central axis

of the stopper and on its lower surface, is attached a glass cross, suitably warped, to which a seed may be fastened with the aid of a sulfur-free rubber band (Fig. 1).

The manometer is of the usual type (Oppenheimer, 1928, Fig. 299) with only one slight modification. The arm of the manometer which joins the vessel has an additional 90° bend near the end, away from the manometer proper, so that when the two are joined, the axis of the

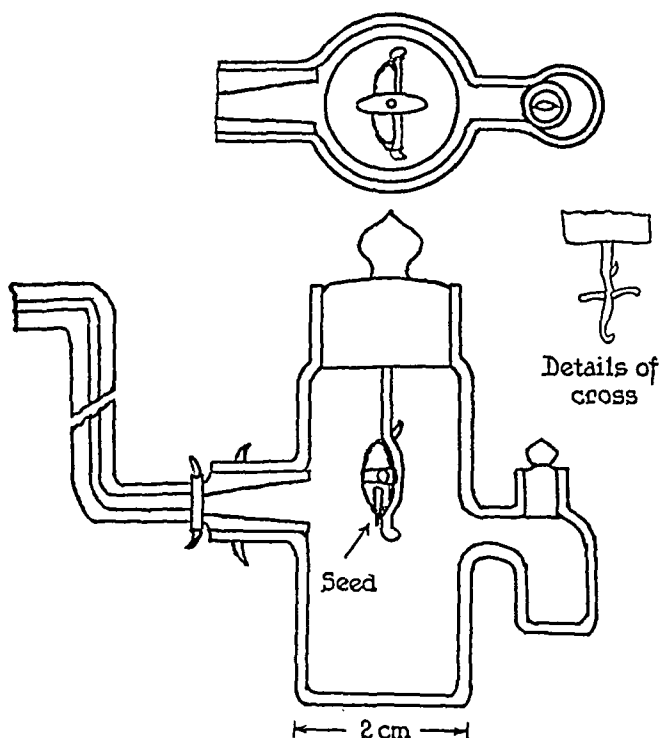


FIG. 1. Side and top views of the vessel used for the study of metabolism of single seeds. Details of the cross are also shown.

vessel is parallel with the manometer, and is parallel to the gravitational force. The hooks for the springs holding the vessel and the manometer together are of the usual type on the vessel, but are attached on a ring on the arm of the manometer. The presence of the ring enables the hooks to remain in line with those on the vessel when the latter is turned to any angle.

For calibration of the vessels and the calculation of the vessel constants, see either Warburg's monograph or Oppenheimer's handbook

cited above. The operation of the respirometer is exactly the same as with the ordinary type, with the exception that it is not shaken. This is done for two reasons: (1) shaking itself may cause geotropic or other kinds of stimulation, and (2) it is not necessary; the seed is suspended in moist air (as defined by the vapor pressures of KOH or other solutions in the vessel at the experimental temperature), and the diffusion of gases to and from the seed is not hindered by the presence of a liquid medium as in the case of tissues suspended in solution.

An experiment with geotropic excitation is cited here mainly to illustrate the use of the apparatus. In Fig. 2, B is the control which is

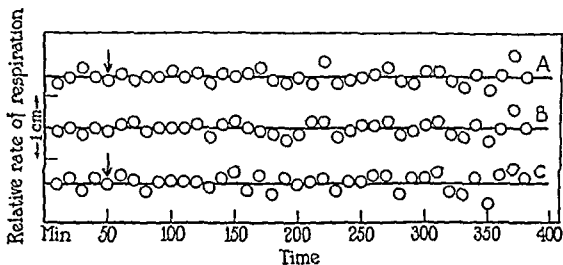


FIG. 2. The course of oxygen consumption by seedlings of *Lupinus albus* before and during geotropic excitation. Ordinate, relative rates of oxygen consumption expressed in terms of Δh Brodie solution/ Δ Time; abscissa, time in minutes. Seedlings A and C are geotropically excited; B is the control. Arrows indicate beginning of excitation.

not stimulated while A and C are turned to an angle of 90° , thus placing the roots at right angles to gravity after the fifth reading (as indicated by the arrows). The rates of consumption of oxygen remained unchanged after stimulation and during the course of reaction. The absence of any rise in rate of uptake of O_2 need not be taken as in discord with the findings of Navez (1928-1929) and of Navez and Crozier (1929) who noticed a rise in the rate of CO_2 production during the geotropic response of *Vicia faba*. The species, the experimental conditions, as well as the phase of respiration, are different in the two cases. It is also highly probable that the seedlings employed here are too young (2 days), and the proportion of the oxygen consumed by the

root is so small compared to that of the cotyledons that even if there were any change, it would have been masked by the larger and constant respiratory rate of the cotyledons, which in extreme cases amounted to 90 per cent of the total respiration.

Details regarding the use of the apparatus for the determination of the respiratory quotient will be found in another paper dealing with the respiratory quotient of the germinating seeds of *Lupinus albus* as a function of temperature (Tang, 1931-1932, *b*).

With electrodes properly mounted through the stopper, the course of respiration of the seed during electric stimulation may be followed.

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ELECTROKINETIC PHENOMENA

VI. RELATIONSHIP BETWEEN ELECTRIC MOBILITY, CHARGE, AND TITRATION OF PROTEINS*

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INTRODUCTION

Solutions of the proteins represent in one sense simple systems, rather than complex ones. The properties of proteins in solutions which depend more or less upon changes in electric charge can be investigated directly through the study of the electric mobility of the protein ions¹ and indirectly by means of titration curves with acids, optical rotation, viscosity, etc. If one makes certain assumptions, clarifying and satisfactory relationships can be obtained, linking the electric mobility, the charge, the titration curves, and the optical rotation of proteins.² The theory and data to be presented permit the statement of the problem more explicitly than heretofore in terms of mobilities, and therefore simplify the approach to many of its real complexities.

Working with gelatin and crude egg albumin it was previously shown that (1):

* Certain of the experiments utilized in this communication have been performed in the Department of Medicine, Johns Hopkins Medical School, and the Department of Physical Chemistry, Laboratories of Physiology, Harvard Medical School.

The data have, in part, been presented at a meeting of the American Physical Society, April, 1931.

¹ Porrett (1816) was the first to show that adsorbed protein was electrically charged.

² Optical rotation is discussed in this connection in the next paper (following) of this series.

"The electric mobility of freely dispersed egg albumin and of microscopic quartz particles covered with the same protein have been shown to be fairly similar between pH 3.2 and 5.5. Zeta-potential measurements by the method of streaming potentials have confirmed this relationship. The mobilities of quartz and other kinds of particles covered with egg albumin and with gelatin have since then been studied in the pH range noted in conjunction with the titration curves for these proteins. Under certain conditions, the change in mobility of the protein covered particles has been found to follow the titration curves of these proteins very closely. All of these observations indicate that the process of adsorption of the proteins mentioned does not appreciably change the ionization of the adsorbed protein at the protein-water interface."

More recently Tiselius (2) has reported experiments on the electrophoresis of dissolved proteins. Tiselius' experiments on the electric mobility of dissolved purified protein by the moving boundary method have now been repeated in part on microscopically visible quartz particles covered with a film of adsorbed protein. The agreement between the results by the two methods is so satisfactory that it is possible to extend the quantitative data for electric mobilities of certain proteins over the range of the acetate buffers, making available, therefore, values of changes in charge over a range of pH sufficiently wide for a preliminary examination of properties presumably dependent upon electric charge. These data on the mobility of adsorbed protein are now presented and discussed in the light of the pertinent theory and of data on titration curves, charge, and optical rotation (3).

Methods

Electric mobility was determined in modified Northrop-Kunitz micro-electrophoresis cells (4). Two cells were employed in the manner described previously (5). Proteins were crystallized and recrystallized as described by Tiselius (2) so that no essential difference in method of preparation could be present. The dialysis of the proteins was accomplished by the method of Abramson and Grossman (6). Electrodialysis was not used. Hydrogen ion activity was measured by means of the quinhydrone electrode and checked with the hydrogen electrode. No corrections were necessary because of the low salt and protein concentrations. Values of pH are referred to the pH of $N/10$ HCl equal to 1.07. Michaelis solutions of acetate buffers were used daily to check all measurements of pH. In general the same concentrations of protein were used in the experiments with quartz particles as in the observations by Tiselius (2); occasionally these were slightly higher. The differences in the results due to different protein concentrations were within the limits of experimental error. Quartz particles were washed

for days with distilled water after cleaning by concentrated HCl and by cleaning solution. Approximately the same number (per unit volume) of quartz particles of the same average size was used throughout. The quartz particles were added to a concentrated protein solution; they remained in contact with the solution for several minutes and were then diluted to the protein and salt concentration required. The acid of the buffer was always added last. A temperature coefficient of 0.02 per degree Centigrade has been assumed to bring all values of the electric mobility, to the same temperature, 20°C., from room temperatures which reached 27°C.

Theoretical

*Potential; Charge; Mobility.*³ The force acting on a sphere having a charge, Q , in a homogeneous electric field, is the product of the charge into the field strength, X . It has been proposed that this relationship be applied to particles moving in aqueous solutions with a velocity, v , to calculate the charge,

$$Q = \frac{6 \pi \eta r v}{X}, \quad (1)$$

η = coefficient of viscosity; r = radius. This concept, applied to aqueous media, is strictly valid only at infinite dilution or when no other ions are present. In ordinary electrophoresis experiments conducted in salt solutions equation (1) does not apply and its use is misleading.

According to equation (1),

$$v \propto Q.$$

This is true for the case of Millikan's oil droplets, but it is not true in general for particles in solutions of electrolytes. In aqueous solutions of electrolytes the potential, ζ , is *not* given exactly by the equation,

$$\zeta = \frac{Q}{Dr},$$

³ In this discussion the general validity and usefulness of the idea of the Helmholtz double-layer is assumed as well as its subsequent developments..

No references are given for the derivations of equations belonging to classical physics. The complete derivations of other equations must be sought in original sources to which sufficient reference is made.

D = dielectric constant of the medium; r = radius of the particle, except for very dilute solutions.

In a medium containing ions the relationship between v , ζ , and Q is more conveniently described through the theory of Helmholtz and Smoluchowski (7), which states that

$$v = C \frac{\zeta DX}{\pi \eta}, \quad (2)$$

C = a constant, and ζ represents the difference in potential between the surface of the charged particle and an equal and oppositely charged layer that can be said to be situated at a certain distance outward in the medium. The velocity of a given particle under changing circumstances is then proportional to the potential difference of the double layer if $\left(\frac{DX}{\eta}\right)$ remains constant. The concept of the double layer has been clarified by the theory of Gouy (8), Debye and Hückel (9), and others (10). See in particular the paper of Müller (10). In accordance with the theory of Debye and Hückel, the potential difference, ζ , is (with certain assumptions), related to the charge, Q ,

$$\zeta = \frac{Q \frac{1}{\kappa}}{D r \left(r + \frac{1}{\kappa} \right)}, \text{ approximately; } \quad (3)$$

where

$$\kappa = \sqrt{\frac{4 \pi e}{D k T} \sum_1^s n_i z_i^2}. \quad (4)$$

($e = 4.77 \times 10^{-10}$ E.S.U.); $k = 1.37 \times 10^{-16}$ ergs *per degree per molecule*; T = absolute temperature; n = number of ions of the type i having the valence z). $1/\kappa$ has the dimensions of distance. If λ is the distance between two concentric spherical shells, the general equation for the potential difference between the shells of a condenser consisting of two concentric spheres is,

$$\zeta = \frac{Q \lambda}{D r (r + \lambda)}. \quad (5)$$

Compare equation (5) with (3). Two assumptions, (discussed in the next paragraphs) have been employed in the derivation of equation (3) and must be borne in mind if this equation is to be used for the calculation of the charge of protein ions. One assumption has to do with the order of magnitude of the values of ζ , the other with the product (κr).

Equation (3) is valid for very small particles only when

$$\frac{\zeta e}{kT} < 1,$$

for uni-univalent electrolytes. The error otherwise involved is that due to the substitution of $\left(\sinh \frac{\zeta e}{kT}\right)$ by $\left(\frac{e}{kT}\right)$ in the solution of the equation

$$\nabla \cdot \nabla \zeta = 4 \pi e \sum_i n_i z_i \sinh \frac{\zeta e}{kT}.$$

For protein ions which have a mobility of about 1×10^{-4} cm., $\left(\frac{\zeta e}{kT}\right) = 0.55$ and $\sinh 0.55 = 0.578$, the difference $\left(\sinh \frac{\zeta e}{kT} - \frac{\zeta e}{kT}\right) = 0.03$ approximately. This assumption can be met more easily by calculating Q still nearer the isoelectric point of the protein, say, where $\left(\frac{\zeta e}{kT}\right) = 0.2$ and $\left(\sinh \frac{\zeta e}{kT} - \frac{\zeta e}{kT}\right) = 0.0013$. In the case of egg albumin and other proteins this use of the method is possible.

An approximation to the way in which the thickness of the double layer depends upon (κr) can be obtained from the theory of the *rigid* double layer. Replacing λ in $\zeta = \frac{4\pi\sigma}{D} \lambda$ for the rigid double layer, by its equivalent in the Debye theory,

$$\zeta = \frac{4\pi\sigma}{D} \left(\frac{1}{\kappa} \frac{\kappa r}{\kappa r + 1} \right),$$

there results (11),

$$\lambda = \frac{1}{\kappa} \frac{\kappa r}{\kappa r + 1}.$$

Evidently if $\kappa r \gg 1$, $\lambda = \frac{1}{\kappa}$. The calculation of Q by means of equation (3) therefore, granting that ζ is sufficiently small, is also interfered with by the fact that the thickness of the double layer depends upon (κr) .

Protein molecules to be considered here cannot be readily regarded as point charges, nor are they very large molecules in the sense of equation (6). The following device can, however, be employed if the change in charge with pH, for example, is to be calculated. This device consists in maintaining (κr) constant. By combining equations (2) and (3) and substituting numerical values for certain constants there is obtained,

$$Q = 6 \pi \eta \sigma r [(r \sqrt{\mu} \times 0.33 \times 10^8) + 1], \quad (7)$$

for unit field strength; $\mu = \frac{1}{2} \sum_1^s n_i z_i^2$, the ionic strength. C is a number which depends upon the size and shape of the particle (for small particles) in a particular electrolyte solution. It seems likely that the value 6 is a very close approximation at present for a sphere the size of a protein molecule in the salt solutions to be considered (12).

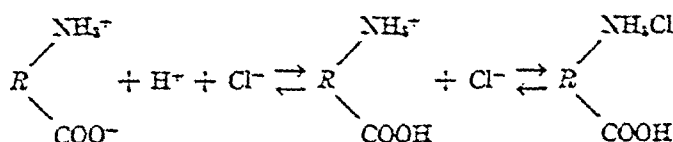
Comparing (7a) with (1a),

$$Q = 6 \pi \eta \sigma r [(r \sqrt{\mu} \times 0.33 \times 10^8) + 1], \quad (7a)$$

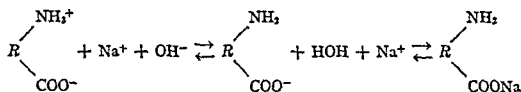
$$Q = 6 \pi \eta \sigma r. \quad (1a)$$

It is evident that the charge calculated by means of equation (1a) can be much too small, for $(r\sqrt{\mu})$ is always positive. Evidently η , r , and μ can be kept constant and a simple relationship obtained for Q and σ from equation (7a). This is discussed in the next section.

Mobility; Charge; Titration Curve.—If a protein in solution combines with an acid or a base, using the zwitter ion concept and schematizing the reaction for HCl and NaOH, the equilibria,



and



describe the important reactions involved. Assuming that no association of the protein salts occurs, and that no other ions combine with the protein molecule, the charge will be exactly equal to the number of hydrogen (or hydroxyl) ions bound. If there is uniform distribution of the charges over the surface of a spherical molecule the potential at the surface in the absence of secondary phenomena will be proportional to the number of hydrogen ions bound. Proteins, however, are always studied at some particular activity of the hydrogen ion and frequently in the presence of salts, that is with $\mu > 0$. It is necessary, therefore, to consider equation

$$Q = 6 \pi \eta v r [(r \sqrt{\mu} \times 0.33 \times 10^9) + 1]$$

in some detail. It is possible to vary the activity of the hydrogen ion and keep the ionic strength sufficiently constant; under these conditions,

$$Q = 6 \pi \eta v r [(Cr \times 0.33 \times 10^9) + 1],_{(C = \sqrt{\mu})} \quad (8)$$

Equation (8) now states the conditions for which any given molecule of radius r moving with a velocity v in a field of unit potential gradient, has the charge, Q , proportional to the electrophoretic velocity. Collecting constants, therefore, from (8),

$$Q = v (C' + C'') \quad (8a)$$

$$C' = (6 \pi \eta r^2 \sqrt{\mu} \times 0.33 \times 10^9)$$

$$C'' = (6 \pi \eta r)$$

Making certain assumptions which immediately follow, equations (8) and (8a) predict the following rule:

In solutions of the same ionic strength, the electric mobility of the same protein at different hydrogen ion activities should be directly pro-

portional to the number of hydrogen (hydroxyl) ions bound by each molecule. This statement includes the following assumptions:

1. The viscosity change with change in hydrogen ion activity is negligible.
2. The effective radius, r , of the protein ions does not change with change in hydrogen ion activity.
3. Either the protein salt is completely dissociated, or what is tantamount here to the same thing, the percentage of dissociation over the range of hydrogen ion activity under consideration is constant for a given protein.
4. The reaction of the protein with ions other than the hydrogen (hydroxyl) ion is negligible.
5. In comparing change in mobility with pH for the same protein, the salt present influences the protein in the same way at all investigated values of pH. (In comparing two proteins, a given salt affects similarly the charge of the different proteins in the range of pH investigated.)
6. Only uni-univalent strong electrolytes are considered.
7. The values of dielectric constant and viscosity of the medium can be used in place of the unknown values in the double layer.

Assumption 1.—In the case of serum albumin and egg albumin, the protein solutions are sufficiently dilute to make any change in η negligible within the limits of experimental error. Gelatin must be described as having its apparent viscosity, η' , composed of two components (13),

$$\eta' = f(F) + \eta$$

where F is the shearing stress and η the coefficient of viscosity. Even though in dilute gelatin solutions η' may be significantly large when compared with η , this increase in η' does not come into importance in electrophoresis measurements in dilute solutions or with soft gels.

Assumption 2.—Svedberg and Nichols (14) and Svedberg and Sjögren (15) have shown that there is no significant change in r (or the equivalent radius) of egg albumin and serum albumin over the range of hydrogen ion activity in the acetate buffers to be considered. Gelatin represents a special case. It will be considered in the experimental section.

Assumption 3.—We are particularly indebted to Hitchcock (16) for demonstrating that certain protein salts are almost completely dissociated from pH 3.6 to pH 4.7. Assumption (3) has therefore a certain amount of experimental justification. This notion cannot be extended to pH's lower than pH 3.5 (3).

Assumption 4.—To distinguish between the pH of the isoelectric point and the pH of pure solution of an ampholyte is not entirely without objection, if this distinction involves an implication that the pH of the pure solution indicates that acid or base is always bound at the isoelectric point. Sørensen (17) showed that the pH of an ampholyte solution must lie between the pH of the neutral reaction and that of the isoelectric point. With increasing concentrations of ampholyte

the pH of the solution approaches the isoelectric point. That this theory holds for proteins has been recently demonstrated experimentally by Hitchcock (18) for gelatin. The pH of gelatin solutions varied with the concentration, approaching to within 0.05 pH of the isoelectric point determined by electrophoresis. In the event that the pure solution of the ampholyte is not at its isoelectric point, addition of acid or base to the protein brings the protein to the isoelectric point as determined by electrophoresis. The only error then involved is due to the presence of a small additional quantity of electrolyte, here negligible, the shape of the titration curve remaining essentially the same.

Assumption 6.—Complications would arise if polyvalent ions were to be used. For example with positively charged ions in the presence of SO_4^{2-} , the titration and mobility curves could not be so simply compared.

EXPERIMENTAL

I. Egg Albumin

1. Electrophoresis and Titration Curve.—The open circles in Fig. 1 are values of electric mobility obtained for egg albumin by Tiselius (2), in $M/50$ sodium acetate-acetic acid buffer from pH 4.27 to 5.27. All the other points are values of mobility obtained for two different samples of egg albumin adsorbed by quartz in the same electrolyte. Note that these protein-covered quartz particles, about 1,000 times the size of the dissolved protein molecules, move with almost the same mobility as the individual molecules themselves exhibit. The dotted line indicates what is probably a slight difference between our data and Tiselius' data. This difference in no way diminishes the rather remarkable nature of the agreement between the macroscopic method employed by Tiselius and the microscopic method.

The heavily drawn smooth curve is *not* a "best" curve drawn through the combined data. It is the titration curve of egg albumin (Loeb (19)), the mols of acid or base bound given on the ordinate. In every respect, in particular in regard to the change in slope, the mobility and the titration curves are congruent within the limits of experimental error, a result in complete agreement with equation (8a). The titration curve has been drawn on the assumption that no acid or base is bound at the isoelectric point, here about pH 4.57. For a given value of mobility a certain amount of acid (base) is bound. This represents a certain fraction of the ordinate. And the same scale is used to plot the remainder of the titration curve. This method of construction of the graphs is not only capable of showing the congruency of the curves,

but it also eliminates smoothing of the data, incongruity being then easily observed. In other words, it is at once evident that if the mobility curves were smoothed, the same equation with different con-

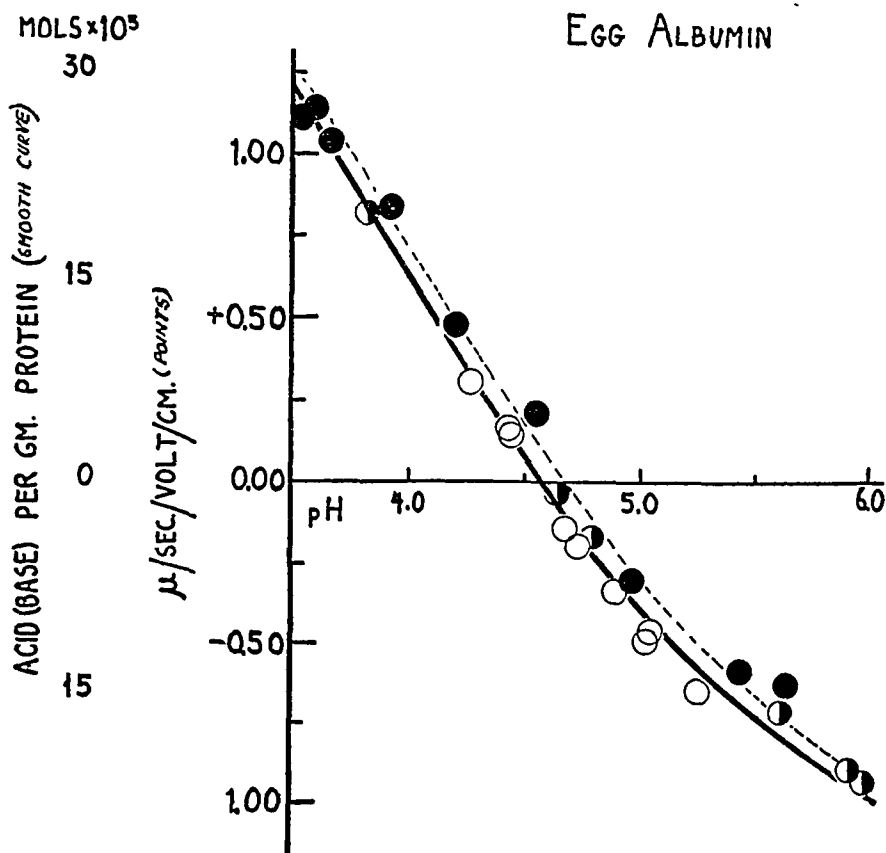


FIG. 1. The open circles are values of electric mobility of *dissolved* egg albumin obtained by Tiselius. The closed and half-closed circles are similar data for egg albumin studied under similar conditions but *adsorbed* on microscopically visible quartz particles. The data of Loeb have been used to plot the titration curve. It is evident that the mobility and titration curves belong to the same family, so that over this range of pH, mobility is proportional to the acid (base) bound. The dotted line indicates the very slight shift in electrophoretic mobility between adsorbed and dissolved protein.

stants would describe both the mobility and the titration curve. That is,

$$v \propto H^+$$

and

$$v \propto (OH)^-,$$

the electrophoretic velocity in solutions of the same ionic strength is proportional to the hydrogen (hydroxyl) ions bound, the rule that has been theoretically derived in the preceding section.

Values of the electric mobility of egg albumin over the entire acetate buffer range given in Table I have been obtained by interpolation from the smooth curve of Fig. 1.

TABLE I
The Electric Mobilities of Two Proteins in M/50 Acetate Buffer

pH	Egg albumin $\mu/\text{sec.}/\text{volt}/\text{cm.}$	Serum albumin $\mu/\text{sec.}/\text{volt}/\text{cm.}$
3.50	1.20	
3.70	0.96	1.30
3.90	0.74	1.05
4.10	0.53	0.79
4.30	0.30	0.55
4.50	+0.077	0.34
4.57	0.00	
4.70	-0.014	+0.16
4.88		0.00
4.90	0.33	-0.044
5.10	0.47	0.27
5.30	0.61	0.39
5.50	0.72	0.50
5.70	0.83	0.63
5.90	0.92	

2. *Denaturation.*—The data contribute some knowledge to the process of denaturation in surface films. It is probable that a polymolecular film of protein is present at the interface of quartz and liquid, first a monomolecular layer and then successive layers being added. The data describe the outermost layer. In this "surface denaturation," if denaturation occurs at the interface (20), except for the extremely small shift of the isoelectric point of about 0.05 of a pH, at the limits of the experimental error, no important change seems to have occurred in the total charge of the outermost molecules of albumin in contact with the liquid. The measurements of mobility were made soon after the suspension of the quartz particles in the protein solutions. It is not impossible that in this type of denaturation the chemical process

is a slow one, and that if a sufficient length of time had been permitted to elapse a more marked change would have been observed. Hendrix and Wilson (21) have determined the amount of acid bound at equilibrium by heat denatured egg albumin. In contrast to our findings on the mobility of adsorbed albumin, it was observed by Hendrix and Wilson that the pH of the protein solution changed appreciably after heating. Heat denaturation evidently can produce changes in the net charge of the protein not so marked in surface denaturation. But according to Booth (22) denaturation can occur without changing the titration curve.

3. *Charge.*⁴—Svedberg and Nichols (14) have found the egg albumin molecule to be spherical and to have a molecular weight of about 35,000. It can readily be calculated from the titration curve, as we have here plotted it, just how many hydrogen (hydroxyl) ions are combined at any given pH. This does not give the number of net charges over that of the isoelectric point unless no negative ions are combined or the protein salt is, so to speak, completely dissociated. Our problem is further complicated by the fact that the titration curves were obtained with the ionic strength changing slightly, for a rather large amount of acid is added, as the ordinate values indicate, whereas the mobility measurements were performed with the ionic strength practically constant. The parallelism of the curves, justifies, however, a procedure attempting to compare the charge Q calculated from measurements of electrophoresis with that calculated from the titration. Assuming complete dissociation of the protein salt, it is reasonable to let the maximum charge be numerically equal to the number of hydrogen ions combined with one molecule of egg albumin. At pH 4.0 (Fig. 2) about 15×10^{-5} mols of H^+ are then combined with 1 gm. of egg albumin. Then each molecule of egg albumin will carry a maximum charge of about 25×10^{-10} E.S.U., over a time average. Let us compare this value with that of Q at the same pH calculated from equation (7a), taking $\eta = 0.01$, $r = 2.17 \times 10^{-7}$ cm., $\sqrt{\mu} = 0.14$, $v = 0.6 \times 10^{-4}$ cm. for $\frac{1}{360}$ volts using c.g.s. electrostatic units, at pH 4.0 in acetate buffer,

$$Q = \left[\frac{6 \pi \eta v r}{X} \right] \left[(r \sqrt{\mu} \times 0.33 \times 10^3) + 1 \right] \quad (7a)$$

⁴ Tiselius has made a somewhat similar calculation.

Using this form of the equation, Q amounts to 14.6×10^{-10} E.S.U. and is about 40 per cent too low.

Although the value of Q calculated from equation (7a) is low, the low value can be expected because the effect of curvature has not been considered. Further analysis aids in clarifying the correlation of titration curve and mobility:

1. By the substitution of the value of Q (25×10^{-10} E.S.U.) obtained from the titration curve into equation (3), an empirical equation can be formulated. This calculation has been made and the equation, corresponding in form to the Debye approximation, is

$$\zeta = \frac{Q}{Dr(\kappa r + 2.4)} \quad (10)$$

Equation (10) yields values of Q (calculated from mobilities) which agree approximately with the values of Q obtained from the titration curve.

2. If equation (6) for the *rigid* double layer is combined with the Debye approximation there is obtained

$$\zeta = \frac{Q}{Dr(\kappa r + 2)} \quad (11)$$

Equation (11) differs from equation (10) only in that 2 replaces 2.4 in the second term in the denominator. This difference is less than the difference between 2.4 and 2.0, for Q , according to this method of formulation, is the sum of two quantities. The value of Q obtained from equation (11) is 22×10^{-10} E.S.U., a value at the limits of experimental error. If equation (6) were written,

$$\lambda = \left(\frac{r}{\kappa r + 1.4} \right),$$

instead of

$$\lambda = \left(\frac{r}{\kappa r + 1} \right),$$

equation (11) would have been identical with equation (10).

It will be of interest to test these equations in more dilute solutions. Experiments of this type are possible. They are simpler to carry out than those now reported. It should be borne in mind that the

combination of the theory of the *rigid* and *diffuse* double layer is not without objection.

II. Serum Albumin

1. *Electrophoresis and Titration Curve.*—Our theoretical rule relating mobility and combined H^+ is confirmed in the case of serum

SERUM ALBUMIN

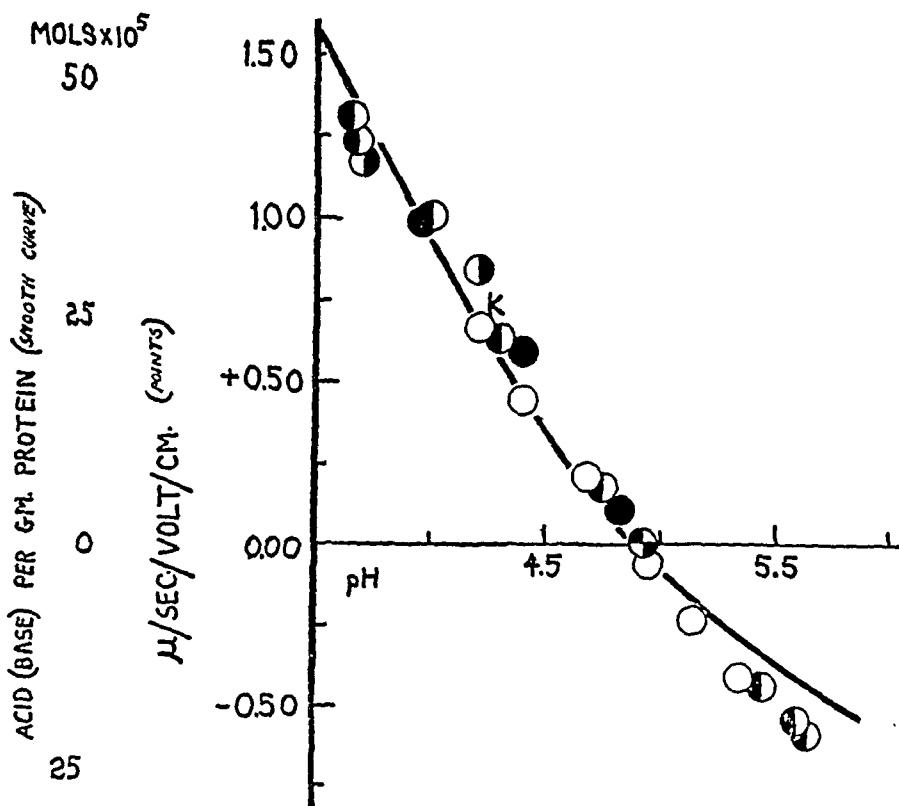


FIG. 2. The open circles are values of electric mobility of *dissolved* serum albumin (Tiselius). The other points are the mobilities of microscopically visible quartz particles covered with an *adsorbed* film of the same protein. There is no difference in mobility between the native dissolved protein molecules and the adsorbed protein. The heavy curve is the titration curve of a sample of serum albumin.

albumin. The open circles in Fig. 2 are values of electric mobility obtained for horse serum albumin by Tiselius (2) in $M/50$ sodium acetate acetic acid buffer. All the other points are values of mobility obtained

here for three different samples of serum albumin adsorbed on quartz particles under the same conditions. The agreement between the electric mobilities of dissolved and adsorbed serum albumin is better than had been anticipated. Within the limits of experimental error there is no difference in mobility in the pH range studied. The heavy smooth curve is the titration curve obtained for a sample of serum albumin, plotted as indicated in the preceding section for egg albumin. The difference between the mobilities observed and those predicted by the theory previously discussed in detail is at the limits of experimental error for mobilities as low as these. Values of the electric mobility of serum albumin given in Table I are from a smooth curve drawn through the combined data.

2. *Denaturation*.—The identity of the values for the electrophoresis of dissolved native serum albumin and adsorbed albumin are in contrast to the data of Pedersen (23). Pedersen has found that serum albumin, after heat denaturation, has an isoelectric point between pH 5.1 and pH 5.3. The mobility-pH curve was nearly parallel to that of the native protein.⁵ If the denaturation occurs at a surface, incidental to adsorption, as in the experiments reported here, this change in the isoelectric point and the mobilities at different values of pH does not occur. There is then, a very great difference now accurately demonstrated between "*surface denaturation*" and *heat denaturation* of the type used by Pedersen, in terms of the charge of the protein.

3. *Charge*.—Since equation (7a) can only be used for spherical particles, it cannot be employed to determine Q with the same validity obtainable for egg albumin. The congruency of the v -pH and the $\pm H^+$ -pH curves indicate, however, that the theory holds. For in the mobility measurements the size and shape of the serum albumin molecules do not vary with pH, so that the experimental finding that $v \propto \pm H^+$ combined, very nearly, is in accord with equation (6a).

III. Deaminized Gelatin

The rule that mobilities are proportional to the number of hydrogen (hydroxyl) ions combined with a protein in solutions of the same ionic

⁵ This had been made the subject of investigation by Michaelis and Davidsohn in 1911 who found the isoelectric point of heat-denatured serum albumin to be shifted from pH 4.7 to pH 5.4. (*Biochem. Z.*, 1911, 33, 456).

strength can be tested in another way. Hitchcock (24) showed that deaminized gelatin adsorbed on collodion particles had an isoelectric point at about pH 4.0, and that acid was bound by the deaminized protein. In Figs. 3 and 4 are plotted the titration curves for Cooper's Gelatin and for the same gelatin deaminized by acetic acid and sodium nitrite. As before, the smooth curves, I and II, are the titration curves for gelatin and deaminized gelatin respectively; our data agree quite well with those of Hitchcock (24) and Simms (25). The closed circles which follow Curve I very well are the electric mobilities of quartz particles covered with gelatin in $N/150$ acetate buffers. Curve I indicates that for gelatin itself, in solutions of the same ionic strength, the mobilities are proportional to the number of hydrogen (hydroxyl) ions combined. Let us assume

(1) that after deaminization the average equivalent radii of the polydisperse protein, deaminized gelatin, is not appreciably changed by the loss of the amino groups;

(2) that the dissociation of the deaminized gelatin in the range of pH studied is the same as for gelatin itself;

(3) that the type of adsorption of both gelatin and deaminized gelatin by quartz particles is the same, and that it represents a mean value of adsorption for a polydisperse system;

(4) that the effective "molecular weight" is unchanged.

Under these conditions, all of which are reasonable, there should be obtained the following relationship:

$$\frac{\text{Combined } (\mp H^+) \text{ gelatin}}{\text{Combined } (\mp H^+) \text{ deaminized gelatin}} = \frac{\text{Mobility gelatin}}{\text{Mobility deaminized gelatin}}$$

That is, in the same buffer, the ratio of acid (base) bound for the two proteins should be equal to the ratio of their mobilities. That this is true experimentally is shown beautifully by the open circles plotted in Fig. 3 along Curve II. These open circles are the mobility values of deaminized gelatin and, as predicted by theory, they fall along the smooth titration curve of deaminized gelatin. Fig. 4 shows the same relationship for partially deaminized gelatin, the isoelectric point of this preparation being at pH = 4.2 rather than at pH 4.0. These two curves represent graphically what happens to the

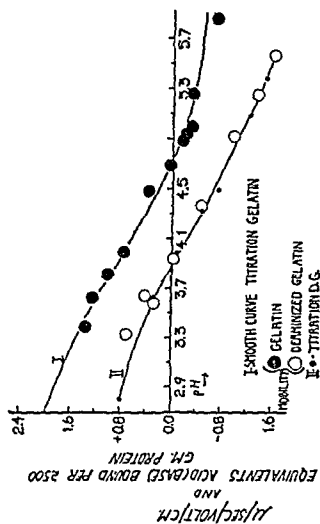


FIG. 3

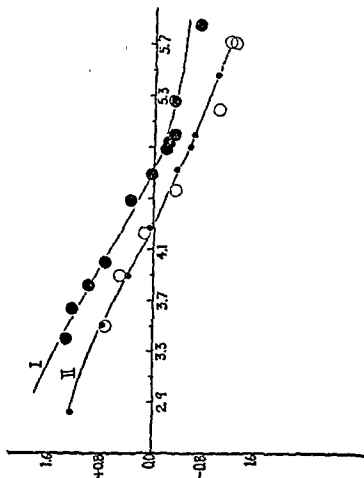


FIG. 4

FIG. 3. In acetate buffer solutions of the same ionic strength, the ratio of the number of mols of hydrogen (hydroxyl) ions bound by gelatin and deaminized gelatin at a given pH is equal to the ratio of their mobilities.

FIG. 4. The experiment and ordinate legend is the same as in Fig. 3. But the gelatin was only partially deaminized. Figs. 3 and 4 demonstrate graphically the congruent changes in charge and combining power of gelatin during the process of deamination.

potential at the surface of the gelatin molecules during the process of deaminization.

In some as yet unpublished experiments with Mrs. J. Daniel, it has been found that alcohol shifts the isoelectric point of gelatin in the reverse direction; in 35 per cent alcohol for example, the isoelectric point is about pH 5.3.

IV. Bence-Jones Protein

This protein, as isolated by Svedberg and Sjögren (26), is monodisperse, and has a spherical molecule of the same radius as egg albumin. It so happens that the molecular weights are also the same, but this condition is not necessary for the validity of the following

TABLE II

Predicted values of acid (base) bound by Bence-Jones protein, isoelectric point pH 5.2, assuming no acid (base) bound at isoelectric point and complete dissociation of the salt. Values are *per gm.* protein.

pH.....	4.3	4.5	4.7	4.9	5.1	5.3	5.5	5.7
Acid (base), <i>mols</i> $\times 10^6$	9.8	7.7	5.5	3.2	0.98	1.3	3.5	5.6

argument. On the basis of the preceding theory and experiments, employing the same assumptions in regard to dissociation of the protein salt, we give in Table II predicted values of the titration curve of the Bence-Jones protein used by Tiselius (2). These predicted values have been obtained by plotting on the same scale the mobilities for egg albumin and Bence-Jones protein (Fig. 5). Then the combined acid (base) has been plotted for egg albumin; if x is the mols of acid bound by Bence-Jones protein, x can be found from the relationship:

$$\frac{\text{Mobility egg albumin}}{\text{Mobility Bence-Jones}} = \frac{\text{Combined } (\pm \text{H}^+) \text{ albumin}}{x}$$

We have tried to check experimentally these predicted values of x , but the sample of Bence-Jones available had an isoelectric point different from that of Tiselius' samples and hence was probably not identical with his protein.

V. Casein

The proteins that have been considered have been soluble in the region of the isoelectric point. For this reason the treatment of the

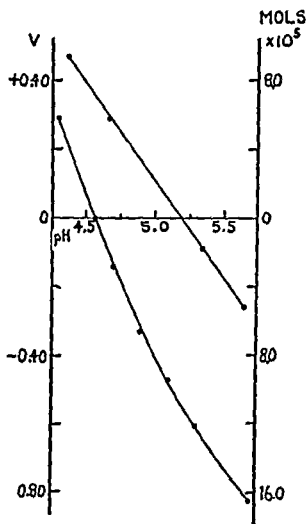


FIG. 5. The ordinate values to the left are electric mobility in μ per sec. per volt per cm. Those on the right are mols $\times 10^5$ of acid (base) bound by the protein. The points of the upper and lower curves are for Bence-Jones protein and egg albumin respectively. The lower smooth curve for egg albumin has been shown to be the same for mobility and combined acid plotted against pH. The upper smooth curve is a *predicted* titration curve for Bence-Jones protein, obtained from the mobilities as described in the text. Table II gives these predicted values in a convenient form.

relationship between combined acid and mobility has been uncomplicated by the insolubility exhibited by a protein like casein in the region of its isoelectric point. Fig. 6, Curve I-I-I, shows the "titration" curve of casein as plotted by Cohn (27). The flat portions of

the curve are in the zone of a heterogeneous system. Loeb (28), on the other hand, pointed out that casein particles are highly charged on either side of the isoelectric point. The slope of the v -pH curve is large and corresponds to those for the other proteins just discussed. Curve I-I-I in the figure represents acid bound for total casein rather

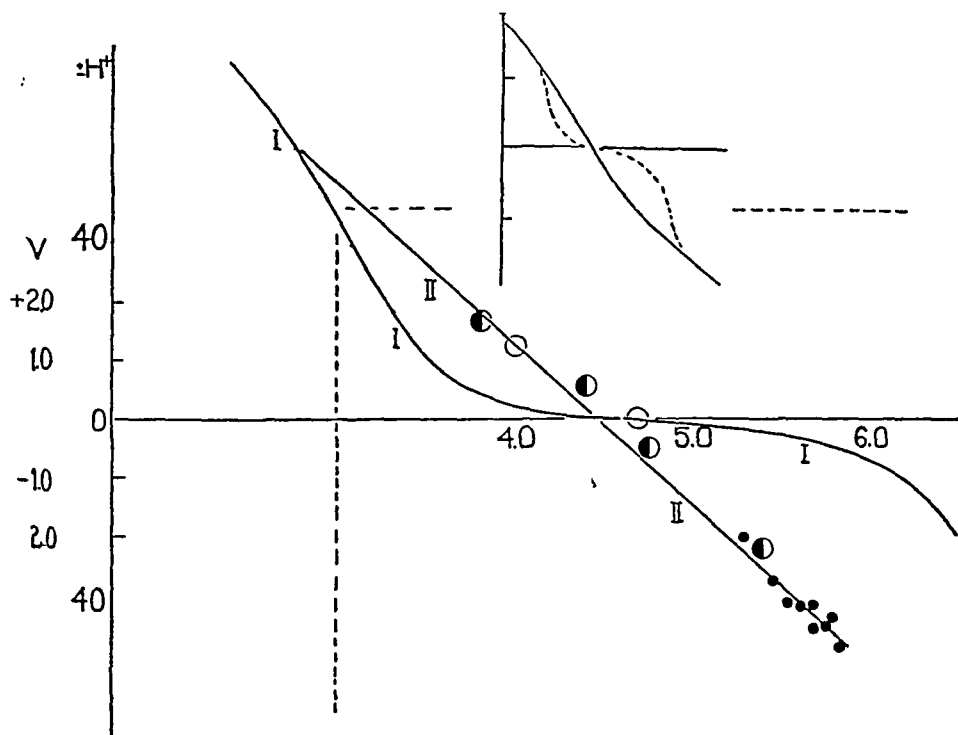


FIG. 6. The open circles (Loeb) and our data (half-closed circles) for the mobility of casein indicate that molecules of casein are highly charged on both sides of the isoelectric point. Calculations of the base bound (closed circles) by casein lead to the postulation of a smooth curve of the sort passing through the closed circles going through the isoelectric point in a linear fashion as indicated in the figure and agreeing in slope with the mobilities. The inset gives a clear picture of the usual "titration" curve (dotted line) and the titration curve here postulated (smooth curve).

than for unit weight of protein dissolved. A serious discrepancy between our approximation and the relationship between combining power and mobility has been solved in the following simple fashion. Data of Cohn (29) have been recalculated so that values of hydrogen (hydroxyl) ion bound per unit weight of casein dissolved have been

obtained. A straight line drawn through the mobility data for casein fits the new titration curve for dissolved casein reasonably well (Curve II-II-I). The slope of the titration curve of casein so plotted, agrees with the slope of the electric mobilities (plotted as before) of casein obtained by Loeb and by us ($\mu \approx 0.005$) in this region and meets the other portions of the curve in a reasonable fashion. These data point to the validity of our rule in the case of casein, and indicate a rational basis for the plotting of titration curves in heterogeneous systems. In this instance the mobility data give a much better index of the change in charge with pH than does the "titration" curve, *unless* combined acid per unit weight of protein is known and the dissociation of the protein. The inset in Fig. 6 perhaps gives a clearer notion of the titration curve of casein as here postulated.

VI. *R-Phycoerythrin and C-Phycocyan*

Tiselius' (2) data include measurements on two spherical protein molecules, R-phycoerythrin and C-phycocyan, having molecular radii of 3.94×10^{-7} cm. and 3.95×10^{-7} cm. respectively, and for the purposes here, equal (30). The combining powers of these two proteins for hydrogen (hydroxyl) ion cannot be as simply predicted as can those of Bence-Jones protein, for the radii are different from that for egg albumin. Tiselius' data were obtained in $M/50$ acetate buffers as in the experiments with egg albumin. Rewriting equation (10), our empirical form of the Debye approximation and collecting constants, there results for a protein molecule of charge Q' , mobility v' , and radius r' , the relationship to egg albumin molecule

$$\frac{Q}{Q'} = \frac{C' v r (r C'' + 2.4)}{C' v' r' (r' C'' + 2.4)} \quad (12)$$

where

$$C' = \frac{6 \pi \eta}{\kappa}, \text{ and } C'' = \kappa$$

(assuming for simplicity that 6 is correct for these spherical molecules differing in radii by the factor 2 although this factor will be slightly smaller for the larger molecules)

or

$$\frac{Q}{Q'} = \frac{v r (\kappa r + 2.4)}{v' r' (\kappa r' + 2.4)}$$

Since for each *molecule*

$$\frac{\pm \text{H}^+ \text{ egg albumin}}{\pm \text{H}^+ \text{ unknown}} = \frac{Q}{Q'}$$

we can calculate the acid (base) bound per molecule by R-phycoerythrin and C-phyococyan from their mobilities, assuming complete dissociation etc.,

$$\frac{\pm \text{H}^+}{\pm \text{H}_z^+} = \frac{v 2.17 \times 10^{-7} [(4.7 \times 10^{-6} \times 2.17 \times 10^{-7}) + 2.4]}{v' 3.95 \times 10^{-7} [(4.7 \times 10^{-6} \times 3.95 \times 10^{-7}) + 2.4]}$$

or

$$\pm \text{H}_z^+ = \left(\frac{v'}{v} \right) (\pm \text{H}^+) 2.27 \quad (13)$$

It is of interest to compare the factor 2.27 in equation (13) with the values of this factor derived from the Debye approximation, 2.57, and from equation (11), 2.33. It would seem that the use of equation (6) in calculations of this sort might not lead to a very large error. In Fig. 7 are smooth curves for the mobilities of egg albumin (Curve 1), R-phycoerythrin (Curve 2), and C-phyococyan (Curve 3). From the mobility and titration data of egg albumin, values of $\frac{v'}{v}$ have been obtained and the acid (base) bound per molecule predicted for R-phycoerythrin and C-phyococyan. These data are given in Table III, and are graphically represented in Fig. 7 by the dotted lines (Curves 2a and 3a). These proteins were not easily available and it is regretted that we can publish only the theory at present. But these calculations clarify the problem and their experimental examination will yield a test of the assumptions made in the application of the theory for molecules differing in radii.

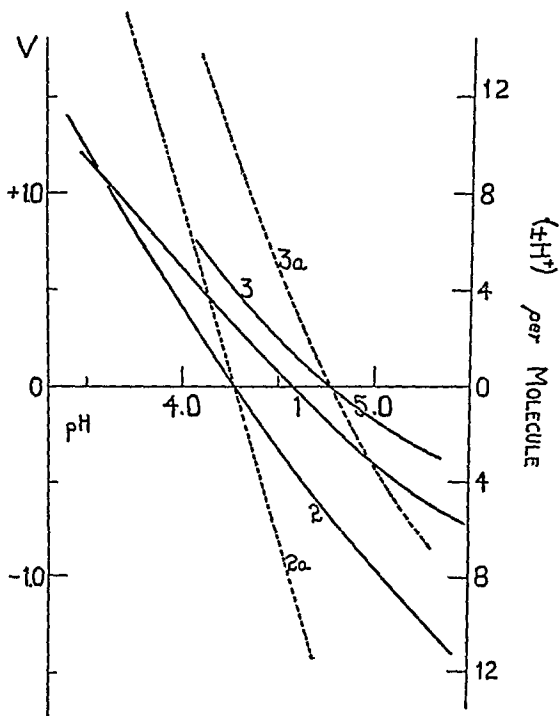


FIG. 7. Curve 1 (Data of Tiselius, Abramson) is the mobility and the titration curve of egg albumin. Curves 2 and 3 are the mobility curves (Tiselius) of R-phycoerythrin and C-phycoerythrin. The dotted Curves 2a and 3a are parts of the predicted titration curves for these two proteins plotted from the data given in Table III, and calculated from the mobility curves using as a reference the data for egg albumin.

TABLE III

pH	<i>Egg albumin</i> ($\pm H^+$) Molecule	$\left(\frac{v'}{v}\right)$	<i>R-Phycocerythrin</i> ($\pm H^+$) Molecule predicted	$\left(\frac{v'}{v}\right)$	<i>R-Phycocyan</i> ($\pm H^+$) Molecule predicted
3.50	10.1	$\frac{124}{118}$	24.1		
3.70	8.1	$\frac{88}{95}$	17.0		
3.90	6.2	$\frac{52}{73}$	10.1		
4.10	4.3	$\frac{26}{50}$	5.1	$\frac{70}{50}$	13.9
4.30	2.48	$\frac{4}{29}$	0.77	$\frac{46}{29}$	9.0
4.50	0.61	$\frac{34}{8}$	5.9	$\frac{25}{8}$	4.3
4.70	1.20	$\frac{60}{13}$	12.6	$\frac{6}{13}$	1.26
4.90	2.70	$\frac{84}{32}$	16.1	$\frac{10}{32}$	1.92
5.10	4.05	$\frac{107}{49}$	20.1	$\frac{24}{49}$	4.5
5.30	5.1	$\frac{129}{62}$	24.0	$\frac{36}{62}$	6.7
5.50	6.1	$\frac{150}{73}$	28.4		

DISCUSSION

Mechanism of Adsorption of Protein.—The fact that not only the isoelectric points but also the absolute mobilities of quartz particles covered with serum albumin or egg albumin are very nearly identical with the values of mobility found for the respective dissolved protein indicates that practically *all* the polar groups of the protein molecules are available even after adsorption has occurred. To demonstrate this let us suppose that one of the hydrogen ions is lost incidental to the adsorption reaction. Near the isoelectric point one H^+ added to each protein molecule gives it a mobility of about 0.10μ per sec. per volt per cm. This very small change can conceivably have occurred in the case of egg albumin, but it is not evident for serum albumin. Since the higher mobilities are practically identical, no change greater than

the loss of one H^+ is probable. In other words, adsorption of a large molecule such as a protein permits practically the full activity of the polar groups to be made manifest in spite of the adsorption. Fig. 8 illustrates schematically what can conceivably occur, the reaction between quartz and protein taking place possibly with the non-polar portion of the molecule. Theoretically a change in the mobility of the protein-covered quartz particles could have occurred also for

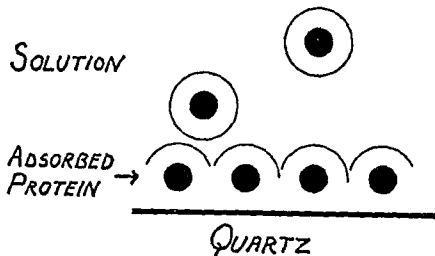


FIG. 8. Schema of proposed mechanism of adsorption of proteins like egg albumin and serum albumin. The protein molecule (central black filled circle) and the outer layer of the double layer (outer circle) are represented without their charges for convenience. Four molecules are adsorbed. Two are free in solution. According to the mechanism here postulated, (1) the adsorbed protein molecules adsorbed have their radii or equivalent radii unchanged. They do not "lie flat" at the interface. (2) The effective thickness of the ion atmosphere about each molecule at the interface is the same thickness as that found for molecules in solution. (3) The available charges are practically the same. (4) The protein molecules determine the nature of the ion atmosphere, the quartz surface playing a negligible rôle at the interface.

the following reason. We have seen that if we utilize the theory of the rigid double layer to give a qualitative picture of what occurs,

$$\lambda = \frac{1}{\kappa} \frac{\kappa r}{\kappa r + 1}.$$

Now r represents more strictly the effective radii of curvature of all points on the surface of the protein molecules or of the quartz particles. To have the protein-covered quartz particles possess mobilities identical with those of individual molecules, it seems necessary that (κr) remain

unchanged, each molecule on adsorption taking its own (κr) along with it; for $v = f(\kappa r)$ and (κr) would vary sufficiently to affect v if any important change in r occurred. In calculating Q for protein-covered quartz particles it is necessary to know the radius of the spherical molecules themselves. The bulk radius of the microscopically visible quartz particles is then probably not the mean radius of curvature of the surface. The calculation of Q for blood cells, bacteria, and other microscopically visible particles will always be complicated by the difficulty of ascertaining the effective values of r . If the mobilities are independent of size and shape of the particles, however, and if comparative measurements are made in solutions of the same ionic strength and species, the mobilities are proportional to the charges and a very good idea of the charge can be obtained by means of equation (7a). The reasoning in regard to (κr) for surfaces in general leads to the establishment of criteria which are necessary for the complete identity of surfaces. It is necessary that not only the chemical (atomic) structures of two surfaces be identical and not only (κr) but also κ and r for each. Identical surface density of charge does not mean identity of surface properties. To illustrate this point imagine a protein molecule having $r = 2.17 \times 10^{-7}$ cm. and a smooth surface, growing larger and larger to say, $r = 1 \times 10^{-4}$ cm., its charge density remaining constant, and the surface still retaining its smoothness; for $\kappa = 0.33 \times 10^7$, utilizing the theory of Henry (12) it can be readily shown that the mobility of the larger particle should be very much greater. Conversely, if the ζ -potential of two different surfaces is the same, the effective radii of curvature of the surfaces may be producing changes bringing chemically different substances to the same ζ -potential.

Activity of Adsorbed Invertase.—The fact that adsorption need not involve certain properties of the polar groups of large molecules simplifies the explanation of a phenomenon observed by Nelson and Griffin (31). These investigators found that, under certain circumstances, *adsorbed* invertase did not lose a significant portion of its enzymatic activity. This is in complete harmony with the facts discovered relative to protein adsorption. It is easily conceivable that enzymes that are protein-like in nature could be adsorbed without diminishing either the number of the enzymatically active groups or the activities of these groups.

The Validity of the Mass Law.—The fact that the same values have been obtained for mobilities of molecules dissolved in a homogeneous system and of molecules existing at a phase boundary indicates that the mechanism of adsorption *per se* need not change the properties of the reactive groups. It could have been anticipated that the forces at a phase boundary would have disturbed the dissociation equilibria, yielding different apparent dissociation constants. This has not occurred. This idea has been developed by Michaelis (32) in connection with the enzymatic behavior of invertase.

The Action of Immune Sera.—Shibley (33) has shown that bacteria treated with immune sera have electrophoretic velocities practically equal to that of serum globulin particles. The reaction of the bacteria with specific groups belonging to serum globulin can occur without disturbing the amphoteric properties of the globulin as the simpler models here studied indicate.

Further Experimentation.—The difficulties of the moving boundary method, in particular the fact that it cannot be used for proteins in dilute salt solutions (2) justifies the experimental extension of data of the type obtainable by the microscopic method employed here. This method can be used over practically the entire pH range usually studied with solutions from infinite dilution to solutions having the conductance of physiological salt solutions. By observance of the principle of having ionic strength and ionic types identical, the properties of the proteins possibly dependent upon their charge can readily be investigated and classified. This is done for optical rotation in the following paper.

SUMMARY

1. By combining the theories of Smoluchowski, Debye and Hückel, and Henry it is possible to state explicitly (making necessary assumptions) under what conditions the following simple rule should be valid for proteins:

In solutions of the same ionic strength, the electric mobilities of the same protein at different hydrogen ion activities should be proportional to the number of hydrogen (hydroxyl) ions bound.

2. Data of Tiselius and of the writer confirm this rule for (a) egg albumin, (b) serum albumin, (c) deaminized gelatin and gelatin, and (d) casein.

3. On the basis of the confirmed theory the titration curves of certain proteins are predicted from their mobilities.

4. It is shown that when certain proteins are adsorbed by quartz the apparent dissociation constant of the adsorbed protein is practically unchanged. The mass law must also be valid at the phase boundary.

5. The facts of paragraphs (1) to (4) are discussed in connection with the mechanism of (a) protein adsorption, (b) enzyme activity, (c) immune reactions, (d) the calculation of the electric charge of cells, and (e) criteria of surface similarity.

I am indebted to several friends for advice received in connection with certain of the difficult problems discussed, especially to Dr. W. J. Crozier for his careful supervision of the manuscript and for his aid in clarifying certain points, and to Dr. J. H. Northrop for his assistance in arranging the data and for an important suggestion considered in connection with assumption (6).

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been reduced to the same wave length, λ , thus eliminating the very wide scatter of his data. This is justified by the linear relationship between α and λ^2 , for then, if the subscripts 1,.....2,.....3,..... n , denote different wave lengths,

$$\frac{\alpha_1}{\alpha_2} = \text{constant, etc.}$$

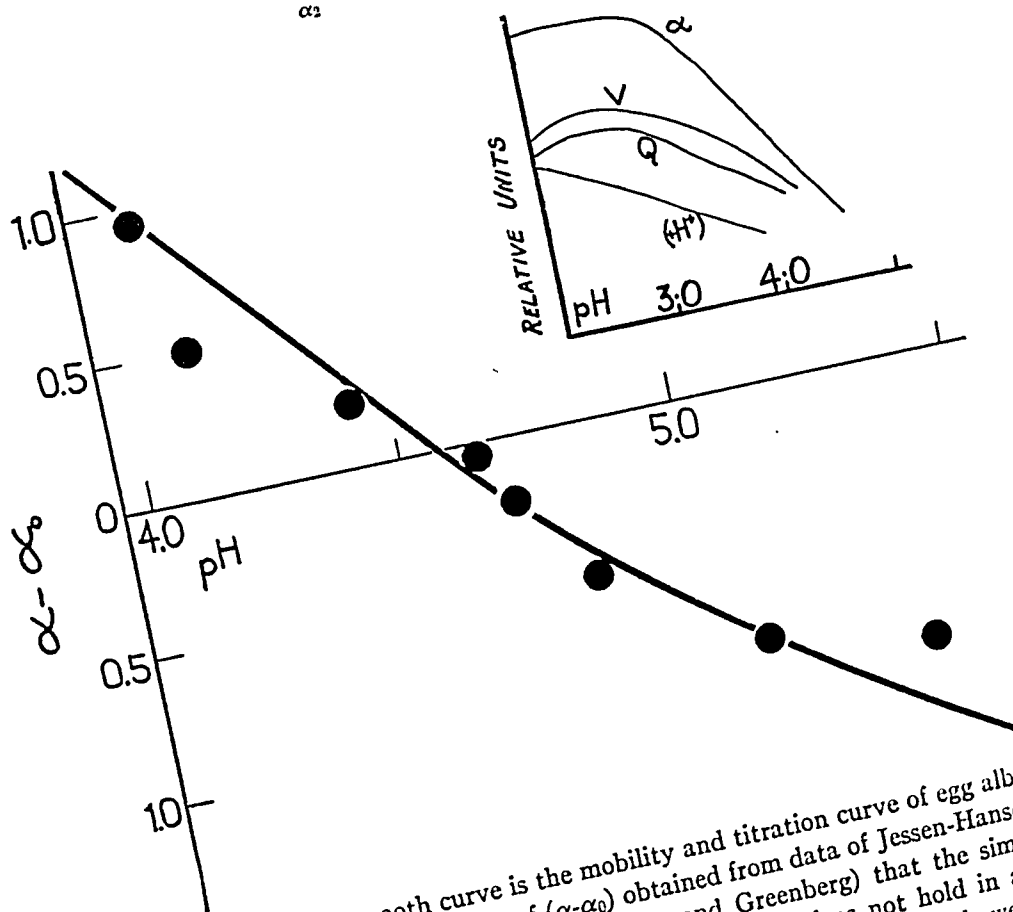


FIG. 1. The main smooth curve is the mobility and titration curve of egg albumin. The points are mean values of $(\alpha - \alpha_0)$ obtained from data of Jessen-Hansen. The inset shows (rotation data from Almquist and Greenberg) that the simple linear relationship between titration and rotation curves does not hold in acid solutions. The mobility (v) and particularly the charge (Q) curves, however, have their maxima nearer the same pH that the maximum rotation is attained.

This conclusion is borne out by an analysis and recalculation of data of Jessen-Hansen. The value of α_0 obtained by interpolation, is then 23.72° , assuming a linear relationship between pH 4.43 and 4.81. The smooth curve in Fig. 1 is our combined mobility and titration

2. *Gliadin*.—The data of Haugaard and Johnson (6) on the optical rotation of gliadin have been calculated on the basis of $\alpha_0 = 115.16$ at pH 7.23. At this point there is a minimum in the α -pH curve. In plotting the data for the relationship between α and acid bound, two different key points were chosen, one for each side of the isoelectric point. Fig. 2 demonstrates for gliadin the congruency of the

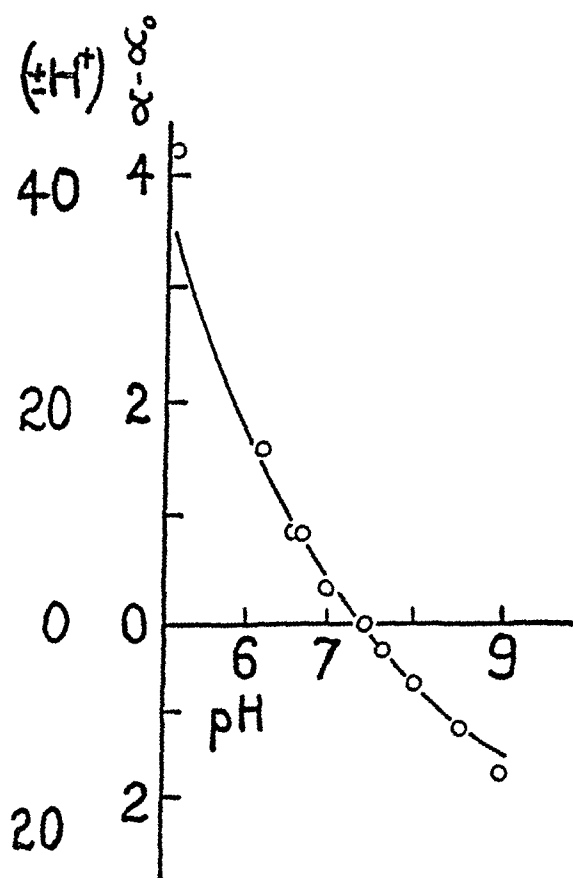


FIG. 2. Data of Haugaard and Johnson have been plotted to show the congruency of the rotation and titration curves in the pH range indicated.

rotation and titration curves from pH 5.1 to pH 8.9 in 54 per cent alcohol. It will be of interest to measure mobilities and rotation and to calculate charge on the same sample of gliadin in solvents having different dielectric constants.

3. *Gelatin*.—In Fig. 3 it is demonstrated that over a wide pH range

a general congruency of the curves of mobility (acid side of isoelectric point) of rotation, of osmotic pressure, and of combining power exist.

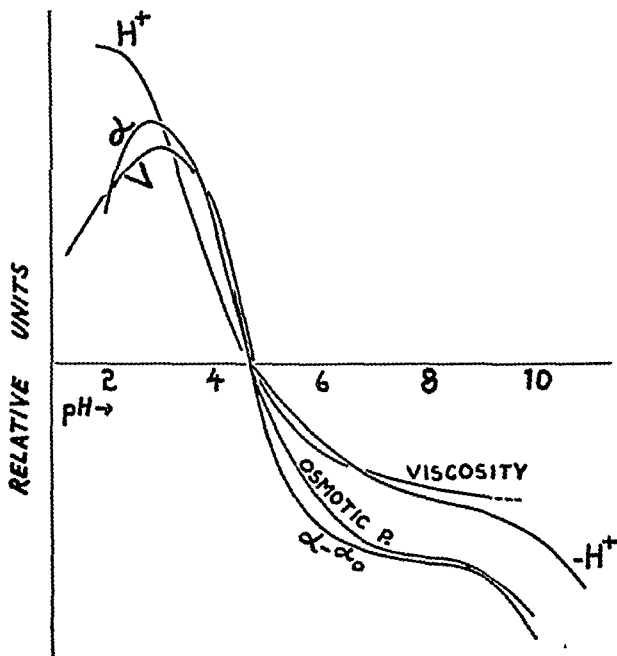


FIG. 3. The changes in optical rotation, osmotic pressure, and $H^+(OH^-)$ bound by gelatin from pH 3 to pH 10 are practically linearly related. The maximum of rotation and mobility observed occurs at about the same pH = 3.0 for egg albumin. The data have been obtained from papers of Loeb, of Hitchcock, and of Kraemer.

The data have been taken from Loeb (7), from Kraemer (8), and from Hitchcock (9). The same maximum occurs in the v -pH and $(\alpha - \alpha_0)$ -

pH curves at about pH 3.0 as noted for egg albumin. Note in particular in Fig. 3 that the combining power, osmotic pressure, and rotation curves all show the same inflection at about pH 9.¹

SUMMARY

The specific rotation of egg albumin, gliadin, and gelatin (40°C.) is discussed in connection with available data on (a) mobility, (b) titration curve, and (c) osmotic pressure. It seems likely that the change in specific rotation with pH of protein solutions is proportional to the change in net charge.

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¹ The viscosity-pH curve is of interest. It can be shown theoretically that the change in viscosity with pH of protein solutions should be proportional to the *square* of the change in charge. That is, in solutions of the same ionic strength the change in viscosity should be proportional to the square of the electric mobility. Data of this sort to test the theory do not seem to be available.

STIMULATION BY THE SALTS OF THE NORMAL ALIPHATIC ACIDS IN THE ROCK BARNACLE *BALANUS* *BALANOIDES*

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EXPERIMENTAL

As it has been demonstrated that closure of the valves, and changes in the cirral rhythm, of the rock barnacle *Balanus balanoides* are reliable criteria of stimulation (Cole, 1932), experiments were made with the salts of the normal aliphatic acids.¹ With a population of 137 animals under constant environmental conditions, the relation between the percentage of closed animals at successive 1 minute intervals and the concentrations of the salts has been determined. The animals were approximately 3 years of age, varying in basal diameter from 8 to 15 mm., and in height from 5 to 12 mm. They were seated on a flat stone, evenly distributed over its surface, and were kept continuously in running sea water for 7 weeks. Temperature was controlled thermostatically at $17.2 \pm 0.2^\circ\text{C}$.; the rate of flow was 250 ± 25 cc. per minute; artificial illumination was constant, and vibrational stimuli were reduced to a negligible minimum. The apparatus was so arranged that the sea water flowing over the animals could be shut off and replaced by the experimental solution at the same rate of flow and temperature, as described in previous papers (Cole, 1928-29; Cole and Allison, 1930-31; 1931-32). The average number of regularly active animals (those showing their normal rhythmic rate) previous to each test was 67, or about 50 per cent of the total. Of the others about 10 per cent were irregular, and about 23 per cent were closed.

From four to nine different concentrations of each of the following acids made up in 3 liters of sea water were used at the same pH as sea water: formic, acetic, propionic, butyric, valeric, caproic, and heptylic, involving 158 tests.² The pH

* A part of the expense of this investigation was met by a grant from the American Association for the Advancement of Science, 1931.

¹ The experiments were done at the Mount Desert Island Biological Laboratory.

² The acids were repurified from Eastman products of the highest purity and their physical constants checked against those given in the International Critical Tables.

was adjusted by adding small amounts of strong NaOH solutions made in distilled water. Since the pH of sea water was always close to 8.1 (± 0.15), the acids were immediately neutralized, forming solutions of the corresponding sodium salts. The number of regularly active animals was recorded at 1 minute intervals for from 8 to 12 minutes, after which no further change in the number occurred. The animals were then thoroughly rinsed in excess sea water and allowed a rest period of 30 minutes before the next test. Reproducible results indicated the complete absence of any adaptive or harmful effects. At the end of the experiments the animals appeared to be in as good a condition as at the beginning, as judged by the rates of cirral movement (Cole, 1932).

RESULTS

For analyzing the data obtained it was assumed that there was a normal distribution of thresholds among the animals previous to each test. During the first interval the animals with the lowest threshold closed, during the last interval those with the highest threshold closed, and during the intervening intervals closure occurred progressively according to the intermediate thresholds. The percentage of animals closing in any interval, therefore, became a measure of the effectiveness of the solution tested, and was calculated on the basis of the number of animals open at the end as compared to the number open at the beginning of the test. Distribution plots of the percentage differences for successive 2 minute intervals were constructed for each concentration of each salt, and the areas enclosed by the plots were calculated graphically. The presence of a symmetrical mode in each case with a decrease in percentage on either side supports the assumption that the thresholds are distributed normally in the population. By plotting the areas against concentration a graphical representation of the effectiveness of the solutions was obtained. Such plots were made for 2, 4, 6, and 8 minute intervals, and in spite of minor differences appeared to be essentially alike, especially the 4, 6, and 8 minute plots. Fig. 1 reproduces the 8 minute plot which will serve as a typical illustration, and furnishes the evidence for the following discussion. Similar relationships appeared when total per cent closure at the end of the successive 2 minute intervals was plotted against concentration.

For the seven heptylate solutions (from 0.0008 to 0.004 M) maximum closure was always obtained at the end of from 30 to 60 seconds. In

spite of continued exposure to the solutions recovery (opening of the valves) began immediately and continued until the end of the 2nd or 3rd minute, when about 50 per cent of the animals had opened again. From then on the rate of closure followed the same course as for the other salts. A similar effect, but less marked, was noted also in the

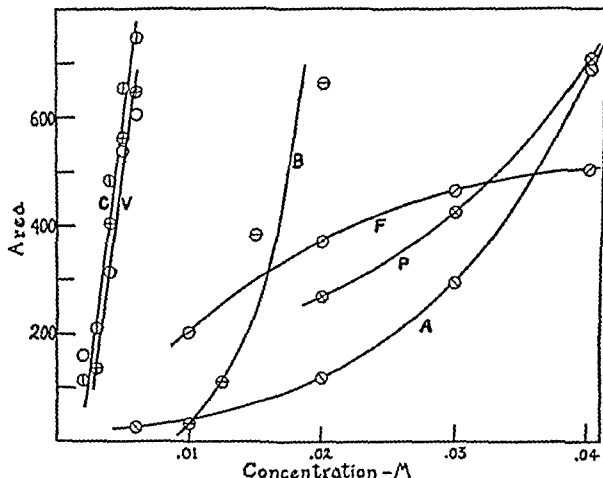


FIG. 1. Effectiveness of solutions of fatty acid salts as stimulating agents on the barnacle, as measured by the areas under the plots of percentage difference in the number of animals closed at the beginning of the test and at the end of 8 minutes, plotted against molar concentration. *F* = sodium formate; *A* = sodium acetate; *P* = sodium propionate; *B* = sodium butyrate; *V* = sodium valerate; *C* = sodium caproate. The open circles represent the values of sodium heptylate, for which no line is drawn. Temperature, $17.2 \pm 0.2^\circ\text{C}$.

higher concentrations of valerate (from 0.003 to 0.01 M). It was absent entirely in the more dilute solutions (0.002 and 0.0025 M) of valerate and in all solutions of the other salts. Although no interpretation of this rapid recovery is offered, its regular occurrence deserves mention.

Fig. 1 shows that effectiveness of the solutions increases with the concentration for all the salts, but that the ratios of effects at increasing concentrations differ for each salt. Omitting the special case of formate, the other salts show an increasing effectiveness with length of carbon chain. The curves become progressively steeper, until the relationship is practically linear for the last three salts. Heptylate appears to be no more effective than caproate, however. Spacing of the plots along the x axis is not logarithmic, indicating that the ratio of effectiveness in respect to length of carbon chain is not a simple exponential function as was found for the alcohols (Cole and Allison, 1930-31). The plot further shows that as the concentration of acetate and propionate increases, their effectiveness converges in such a way that at about 0.04 M they are equal. The formate curve is different from the others in being concave to the x axis, and in having a much lower gradient. For butyrate the effectiveness increases remarkably more than for either acetate or propionate; and for the valerate, caproate, and heptylate the increase is still more marked. In other words, the range of concentrations within which there is a graded response correlated with concentration is largest for formate and steadily decreases up to valerate. Qualitatively this is similar to the results obtained from the alcohols.

Since the area plotted along the y axis is a measure of the effectiveness of each solution, it is possible to read from the plot the concentrations which are equally effective. From the plot of Fig. 1, and from the plots of total percentage closure against concentration, equally effective concentrations were interpolated for areas of 400 and 600, and for 40 per cent, 50 per cent, and 60 per cent closure. Excepting the 60 per cent closure effect, the interpolated values for any given effect were nearly constant, and they were therefore averaged. Fig. 2 shows the plot of these averages against the number of carbon atoms in the chain (continuous line), and the concentrations necessary to produce 60 per cent closure (dotted line). The concentrations for the first effect are: formate 0.0183; acetate 0.032; propionate 0.028; butyrate 0.0153; valerate 0.0044; caproate 0.0037 and heptylate 0.0037 M . In general it may be said that to produce a given effect which will not exceed 50 per cent closure, formate is more effective than acetate and propionate, and only slightly less effective than

butyrate, but considerably less effective than any salt higher in the series. Beginning with acetate the effectiveness increases quite regularly with length of carbon chain up to and including valerate. If a greater effect is selected as a criterion (*i.e.*, 60 per cent closure) formate also falls into the series, being much less effective than acetate

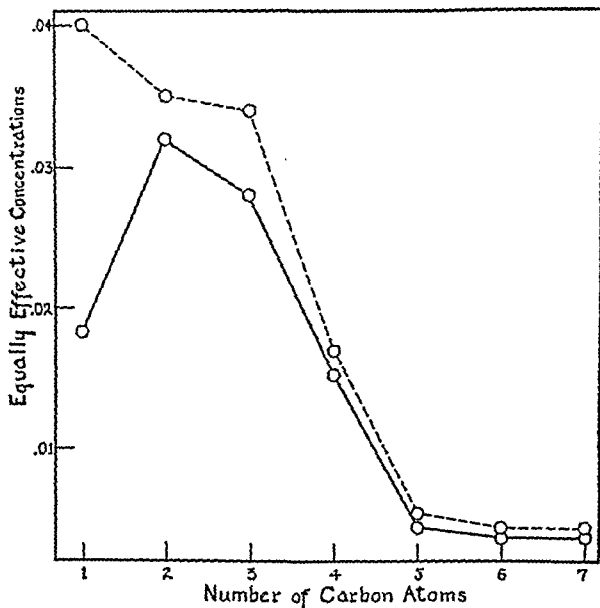


FIG. 2. Concentrations of the seven salt solutions which were equally effective plotted against the number of carbon atoms in the respective molecules. The solid line is drawn through the average concentrations as interpolated from the plots of area (400 and 600) *vs.* concentration and the plots of 40 per cent and 50 per cent closure for the 8 minute interval. The dotted line connects the concentrations as interpolated from the 60 per cent closure plot for the 8 minute interval.

(dotted line, Fig. 2). In measuring the effect of these salts upon the barnacle therefore special attention must be given to the criterion indicative of the effect. A relationship similar to each of those mentioned above has been previously demonstrated for sea urchin eggs by Loeb (1908-09), and for the earthworm by Crozier (1916).

Measurements of the surface tension at an air-sea water interface were made by the Harkins' drop weight method (Harkins and Brown, 1919; 1929), at $17.2 \pm 0.2^\circ\text{C}$., of sea water, of the equally effective

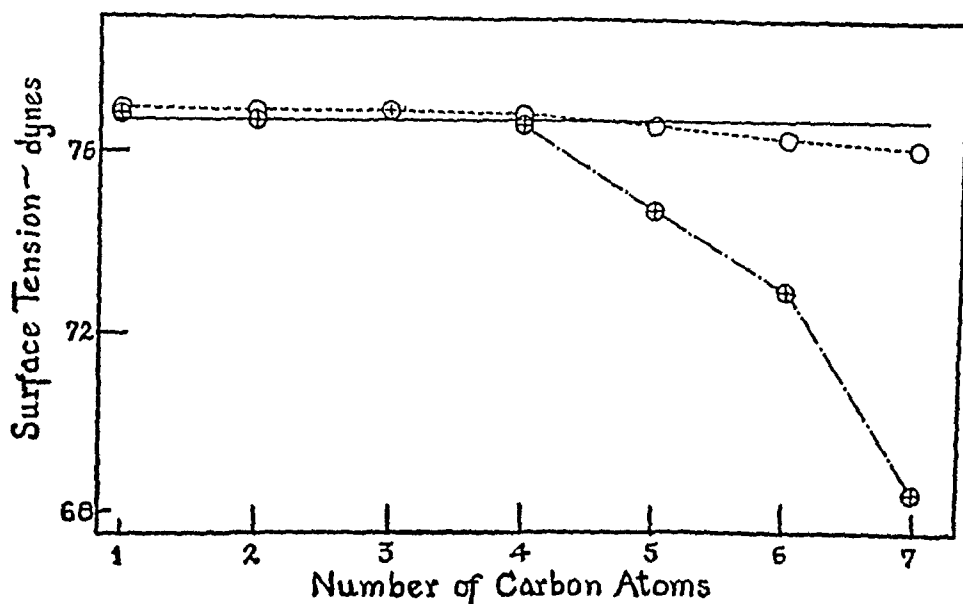


FIG. 3. Surface tension in dynes for each of the seven salt solutions plotted against the number of carbon atoms in the respective molecules. The solid line represents the surface tension of sea water; the dotted line connects the surface tension values for the equally effective concentrations along the continuous line of Fig. 2; the dashed line represents the surface tensions of the 0.028 molar solutions. Temperature, $17.2 \pm 0.2^\circ\text{C}$.

concentrations of the seven salt solutions, and of each salt solution at the same concentration (0.028 M). These data are represented in Fig. 3, showing that for the first four members of the series the surface tension is constant and only very slightly greater than that of sea water. For the equally stimulating concentrations the surface tensions of the higher members tend to decrease very slightly, but for the same concentrations (0.028 M) throughout the series, the surface

tension decreases noticeably beginning with valerate. The significance of these values will appear in the following discussion.

DISCUSSION

It is assumed that the reaction measured is a function of the intensity of stimulation of the receptor surface; that it is reversible, and that it may be quantitatively correlated with the available energy of the particles initiating the series of events called stimulation in the heterogeneous system of environment and receptor. Among the normal primary aliphatic alcohols previously studied the potential of the polar group was considered constant throughout the series and was held to function primarily as an orienting group. The stimulating efficiency of successive members of the series was then correlated with the non-polar portion of the molecule and its power to initiate changes at the receptor interface (Cole and Allison, 1930-31). In the normal aliphatic acid series the potential of the polar group of propionic acid and all the higher members of the series is constant, roughly the same for acetic, but considerably different for formic (Langmuir, 1929). That this higher potential of the formic polar group must be considered in stimulation of some receptors has been demonstrated for the earthworm by Crozier (1918), and for the sunfish by Allison (1931-32). To test the stimulating efficiency of the charged anion resulting from the dissociation of the aliphatic acids, it is necessary to use their salts, thereby eliminating any effect of the hydrogen ion. Equal degrees of dissociation for each salt may be assumed. The greater attraction of the polar group of the salts for water reduces the tendency of those salts to concentrate at an air-water or an air-oil interface. In fact, at an air-water interface the first few members of the series tend to increase the surface tension slightly when used over a certain range of concentrations. As the length of the carbon chain increases, however, the tendency to concentrate at the interface increases until lowering of the surface tension is the predominant effect (*cf.* Fig. 3). At an air-oil interface lowering of the surface tension is the predominant effect from acetic acid up, but the differences between the lower members are slight in dilute solutions (Donnan and Potts, 1910). If stimulation by the sodium salts of the fatty acids were directly correlated with the tendency of the anion to concentrate or to orient at the

receptor surface, and if the polar group acted primarily as an orienting group, then little difference would be expected between the effectiveness of the first members of the series. As the length of the carbon chain increased the tendency of the anion to orient at the interface would become dominant, and stimulating efficiency would be correlated with the length of the carbon chain. If the potential of the polar group were the only factor to be considered, then sodium formate would be more efficient than sodium acetate, but the higher members of the salt series would not differ much from the acetate, regardless of the length of the carbon chain. A relative measure of the potentials of the polar groups in a homologous series, such as the fatty acids, may be obtained by comparing the ionization constants of the members of the series (*cf.* Langmuir, 1929, above). The pK for formic acid is less than that for acetic acid. For acetic acid the pK is slightly less than for propionic acid, but for the next five acids used in the experiments the pK is practically constant. This means that sodium formate would hydrolyze less than acetate; the acetate slightly less than the propionate, but for the next five salts the amount of hydrolysis would be practically constant. In other words, in a series of equal concentrations there would be less free formic acid in the formate solution than free acetic acid in the acetate solution and a higher concentration of the formate anion than of the acetate anion. Similarly in the acetate solution there would be slightly less free acetic acid than free propionic acid in the propionate solution and a slightly higher concentration of the acetate anion than of the propionate anion. For the next five solutions the amount of free acids and the concentration of the anions would remain practically constant. The same sort of relationship has been demonstrated between the electron sharing ability of the organic radicals in the fatty acids and their ionization constants (Hixon and Johns, 1927).

In sea water solutions of the sodium salts of the fatty acids the dissociated anion of the salt is abnormal to a marine environment in the sense that it is usually absent. Even though the salt has no effect upon an air-water interface, or raises instead of lowers the surface tension because of the repulsion of the non-polar portion of the anion by water, there would be a definite tendency to replace other anions near the surface. If the receptor interface is altered by such

replacement, there would be some correlation between length of carbon chain and stimulating efficiency, since as the number of CH_2 groups increases the anion concentration at the interface would increase, thereby shifting the dynamic equilibrium at the interface. In stimulation of the barnacle by salts of the fatty acids, then, both the potential of the anion and the concentration of that ion near or at the receptor interface as determined by the length of the carbon chain, must be considered (*cf.* Cole and Allison, 1930-31). Sodium formate is more effective than sodium acetate because of the greater potential and mobility of the formate ion. Propionate is about as effective as acetate, being only slightly more effective than acetate, but less than formate. Beginning with butyrate the length of the carbon chain begins to play the predominant rôle, so that the stimulating efficiency of the higher members progressively increases as the number of CH_2 groups increases. The maximum effect is reached, however, in caproate, since heptylate shows no increased effect. If the formate is used in high enough concentration (*i.e.*, to produce 60 per cent closure), then it is less effective than acetate, a result correlated with the length of the carbon chain, and the tendency of the anion to concentrate at the interface.

SUMMARY

1. Stimulation in the rock barnacle *Balanus balanoides* by the sodium salts of the first seven normal aliphatic acids has been studied at several different concentrations for each salt. The pH was adjusted to that of sea water (8.1 ± 0.15) and all experimental conditions were held as constant as possible. Criterion of response was the per cent closure of valves at successive 2 minute intervals.

2. In general, the stimulating efficiency increases with concentration, but the ratios of effectiveness at increasing concentrations differ for each salt.

3. The order of effectiveness for 40 to 50 per cent closure is: heptylate = caproate > valerate > butyrate > formate > propionate > acetate. For 60 per cent closure or more, formate is the least effective of all.

4. Stimulating efficiency is correlated with the potential of the anion of the acid and with the concentration of that ion near or at the receptor surface as determined by the length of the carbon chain.

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STIMULATION BY HYDROCHLORIC ACID AND BY THE NORMAL ALIPHATIC ACIDS IN THE SUNFISH EUPOMOTIS

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A study of chemical stimulation in aquatic animals involves a correlation between the chemical nature of the compound under consideration and the changes its presence may produce upon the responsive organism and upon the chemical environment. In previous papers (Cole and Allison, 1930-31; 1931-32) probable effects of the structure and potential of very reactive groups as well as the structure and potential of less reactive groups of the stimulating compound have been considered in a general way. Homologous series of organic compounds have been and are being studied because of the better known variation of their chemical and physical properties as the number of CH_2 groups increases. Such a study it is hoped will lay the foundation for an investigation of more complex series of related compounds.

It has been assumed that the response measured is a function of the intensity of stimulation of the receptor interface, and that whether the reaction is best related to an interfacial tension change, or to a redistribution of charged particles at the surface, or to some unknown chemical situation in the receptor itself, it is, however, reversible and may eventually be related to the chemical nature of the substance which initiated a series of events in the heterogeneous system. For example, stimulation by the normal aliphatic alcohols has been related to the length of the carbon chain. It may be assumed that an energy

* Part of the expense of this investigation was met by a grant-in-aid from the National Research Council (1932) to which grateful acknowledgment is hereby made.

change is produced at the receptor interface which is initiated by the spreading of alcohol molecules at the interface, and that this spreading is influenced by the chemical nature of the receptor as well as by its chemical environment. A shift in the ionic equilibrium at the interface would also follow. Although other factors may eventually be identified as playing rôles in stimulation by alcohol, including the efficiency of the neuro-muscular mechanism of the animal itself, it is clear that the reaction measured may be correlated with the non-polar portion of the molecule and its power to initiate energy changes at an interface. The effect of the polar group in the alcohol molecules appears constant, and it may be said to function primarily as an orienting group. In fact, the effect of an organic molecule upon the tension of an air-water interface may be considered as a measure of the potential and structure of the non-polar group as well as a measure of the orienting properties of the polar group which in turn are related to its chemical nature. However, as in the case of reactions at any interface, the structure and chemical nature of the receptor may modify or change the relationships between the efficiency of a series of compounds. For example in the isomeric alcohols, stearic hindrance may modify the reaction, and in the aliphatic acids the polar group may play a more or less active rôle in shifting the dynamic equilibrium at the receptor interface. The data presented in this report deal with the latter problem.

Methods

Fresh water sunfish, *Eupomotis gibbosus* Linnaeus, of uniform size (approximately 3-4 cm. in length) were kept in separate containers in spring water. The apparatus and technique used in the experiments were similar to those used for the studies on the catfish *Schilbeades* (Cole and Allison, 1931-32). An individual was transferred to the reaction chamber so that the flow of spring water (100 cc. \pm 5 cc. per minute) passed in the antero-posterior direction in reference to the fish. Except for a few exceptions, 10 minutes were allowed for adaptation of the fish to the container. The flow of spring water was then turned off and the experimental solution turned on at the same rate. The reaction observed consisted in cessation of mouth movements or in a marked change in rate which may be best expressed by the word "gulping." The reaction time was measured with a stop-watch to within 0.1 seconds. The response is definite and characteristic and may or may not be accompanied by bodily movements. The latter were not timed. The animal was then thoroughly washed with spring water and transferred for several

minutes to a large dish containing fresh spring water before returning it to the original container. Eight fish were tested in this way in succession and at no time were more than two reactions taken on one fish on 1 day. Usually 1 to 2 hours elapsed before the fish was used for a second experiment. The spring water and experimental solution were brought to the same temperature by passing them through a Pyrex coil 4 meters long immersed in a water bath kept at $18.0 \pm 0.1^\circ\text{C}$. The acids were purified and fresh solutions were made in spring water the day they were to be used. The (H^+) was measured with the quinhydrone electrode. The pH of the spring water varied from pH 6.4 to 6.8.

In his study on sensory activation of the earthworm by alkalies Crozier (1917-18) reduced the average reaction time by a constant which gave him a measure of the true retraction time. The significance of the data on the sunfish was increased by subtracting 5 seconds from the average reaction time for each concentration. The value subtracted here may be considered as an approximation of the shortest reaction time possible without producing toxic effects under the above experimental conditions. "Reaction time," hereafter will refer to the corrected values.

RESULTS AND DISCUSSION

In a previous paper (Cole and Allison, 1931-32) it was pointed out that a receptor which is susceptible to a slight change in (H^+) of its chemical environment might appear equally affected by inorganic or organic acids if the concentrations needed were so low that other disturbances related to the activity of the anion, or to the non-polar portion of the organic molecule, had little or no effect. The statement was also made that in the case of normal aliphatic acids, "as the length of the carbon chain increases the non-polar portion of the molecule might begin to play a stimulatory rôle and with the higher members of the series, it might predominate." It is also evident that certain receptors under proper conditions might react to a combination of such forces. A study was made on stimulation by hydrochloric acid in the catfish *Schilbeodes*, and the conclusion was reached that the reactions observed could be correlated with the potential of the cation. The data obtained on the sunfish lead to the same conclusion, except that this animal is much less susceptible to a change in the (H^+) of its environment and shows also (as might be predicted) more susceptibility to the changes initiated by the non-polar portion of an N aliphatic acid molecule.

It is apparent (Table I, Fig. 1) that when reaction time is related to the pH of the solution in spring water some other factor than the

(H⁺) is involved in the initiation of the response to the *N* aliphatic acids. With increasing length of carbon chain these acids become more efficient as stimulating agents. However, if the logarithm of the concentration is related to the logarithm of the rate of reaction (Fig. 2) formic acid appears much more effective than should be the case if the

TABLE I

Reaction times of the sunfish, *Eupomotis*, to different concentrations of hydrochloric acid and of six *N* aliphatic acids. The number of reactions is 16 except those indicated by a * where it is 15.

Acid concentration-molarity		pH	Mean reaction time	P.E. _M	Acid concentration-molarity		pH	Mean reaction time	P.E. _M
			sec.					sec.	
Hydrochloric acid	0.0049	2.48	2.42	0.184	Propionic acid	0.008	4.13	2.57	0.167
	0.00392	2.50	2.48	0.143		0.005	4.38	3.70	0.215
	0.00294	2.87	3.46	0.127		0.004	4.50	4.04	0.265
	0.00245	2.97	4.36	0.227		*0.0025	4.83	6.30	0.392
	0.00196	3.20	5.60	0.429	Butyric acid	0.004	4.48	2.30	0.137
Formic acid	*0.0149	2.92	2.56	0.179		0.003	4.65	2.99	0.192
	0.00994	3.07	2.43	0.114		0.0025	4.80	3.88	0.208
	0.00745	3.19	3.30	0.214		0.00225	4.92	4.91	0.417
	0.00497	3.35	3.83	0.336	Valeric acid	0.003	4.60	2.34	0.156
	*0.00397	3.43	3.56	0.229		0.00234	4.82	2.57	0.158
Acetic acid	0.011	3.90	2.50	0.236		0.002	5.02	4.10	0.349
	0.01	3.96	2.30	0.200		0.00187	5.00	4.15	0.244
	0.008	4.03	3.39	0.359		0.00174	5.17	4.28	0.321
	0.006	4.20	3.64	0.241	Caproic acid	0.0025	4.8	2.28	0.158
	0.004	4.45	6.66	0.916		0.002	5.08	2.78	0.197
						0.0015	5.40	3.85	0.312
						0.0012	5.89	7.18	0.612

$$P.E. = \pm 0.8453 \frac{\sum (+V)}{n \sqrt{n-1}}$$

reaction involved was primarily one that could be related to the tendency of the organic acids to concentrate at an air-water or an air-oil interface. The ability of the acid to change the (H⁺) of the spring water may be used as one measure of its polar nature. As would be expected, hydrochloric acid is more effective in this respect than formic

(Fig. 2) is less than for the others which results in a differing relationship between this acid and the other members when equally effective concentrations are related to the length of the carbon chain. At the higher concentrations formic acid is less efficient when compared with the other members than it is at lower concentrations. Stimulation of the rock barnacle, *Balanus balanoides*, by the salts of the *N* aliphatic

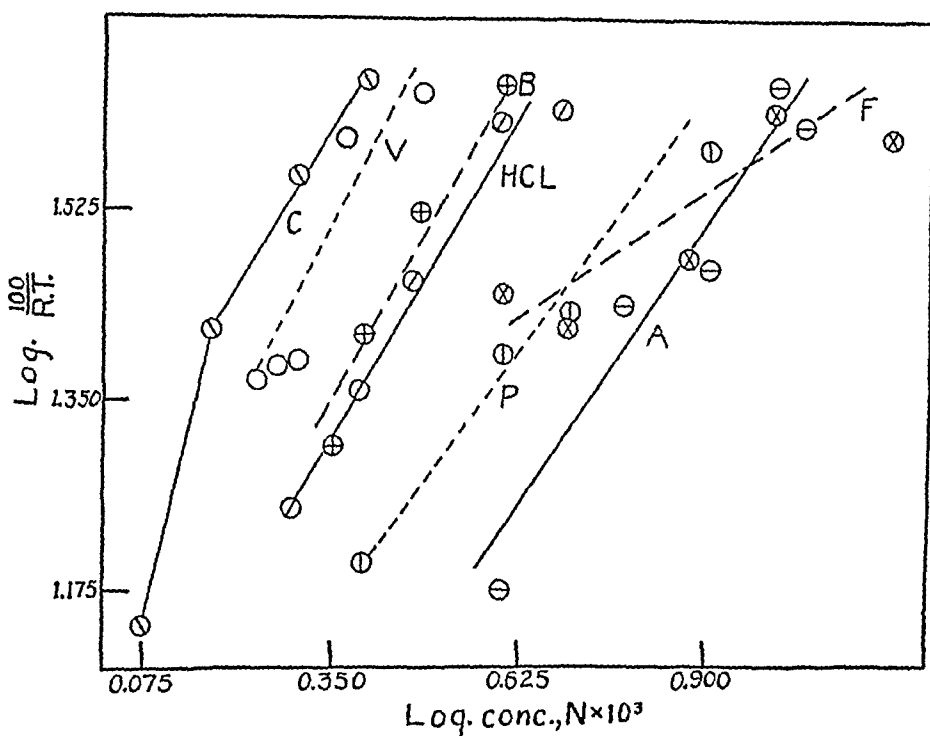


FIG. 2. Plot of the logarithm of the rate of stimulation
 $\left(= \frac{100}{\text{reaction time in seconds}} \right)$
 of the sunfish, *Eupomotis*, to hydrochloric, formic (F), acetic (A), propionic (P), butyric (B), valeric (V), and caproic (C) acids against the logarithm of the concentration ($N \times 10^3$) (data in Table I).

acids (Cole, 1931-32) shows a similar relationship between sodium formate and the other members of the homologous series. With increasing concentration sodium formate becomes less and less efficient when compared with sodium acetate, sodium propionate, etc. This variation may be interpreted to mean that as the concentration of

formic acid is increased the effect upon the receptor interface becomes more of the nature of that produced by the higher members of the series.

It is concluded then that the dominant factor in stimulation of the sunfish by the *N* aliphatic acids may be correlated with the non-polar nature of a portion of the molecule, but that it is necessary to consider the higher potential of the polar group of formic acid to account satisfactorily for its position in the series. Experiments made by

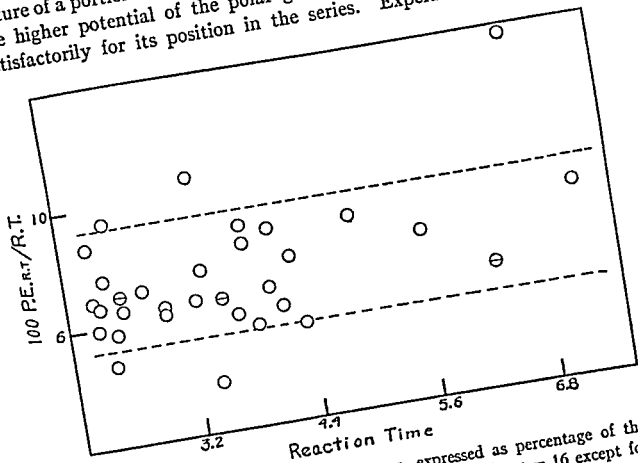


FIG. 3. A mass plot of the probable errors, expressed as percentage of the mean reaction time; against the reaction time. (See text.) $N = 16$ except for three points which are indicated by horizontal lines where it is 15.

Crozier (1917-18) on the stimulating efficiency of the fatty acids on the earthworm, and his interpretation thereof, support this argument.

The reliability of the data which has been presented may be tested in part by utilizing the principles of reaction variation which have been developed by Crozier and Pincus (1931-32, and papers quoted therein). Although it was impossible to be certain that the population of fish selected for these experiments was homogeneous, the probable errors of the mean reaction times do show a consistent relationship to

the variable pH. A series of curves similar to those shown in Fig. 1 are obtained when the probable errors are related to the pH of the solution of each acid. When the probable errors, expressed as percentage of the reaction time, are plotted *en masse* against the reaction time, a horizontal relationship between the two values appears which means that the *per cent variation* is independent of the change in the chemical environment to which the animal responded.

SUMMARY

1. The reaction of the sunfish, *Eupomotis gibbosus*, to different concentrations of hydrochloric acid and of the first six members of the N aliphatic acids has been studied.

2. The stimulating efficiency of hydrochloric acid may best be related to the concentration of hydrogen ions produced by that acid.

3. The stimulating efficiency of the N aliphatic acids may best be correlated with the non-polar nature of a portion of the molecule, but it is necessary to consider the higher potential of the polar group of formic acid to account satisfactorily for its position in the series.

4. When equally effective concentrations of the N aliphatic acids are compared, formic acid is more effective at lower concentrations than at higher.

5. Per cent variation in response appears to be independent of the chemical environment to which the animal responded.

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PORPHYRIN COMPOUNDS DERIVED FROM BACTERIA*

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Cytochrome has been recognized in recent years as a constituent of aerobic bacteria, as well as of aerobic cells generally. The substance which is responsible for the characteristic absorption spectrum is undoubtedly complex and may offer as difficult a problem in its analysis as that of the related pigment chlorophyll. Evidence has been presented, however, that cytochrome has an important share in the pathogenic activities of certain bacteria so that the chemical nature of the pigment becomes a matter of interest (1).

A brief review of cytochrome may not be superfluous in view of the limited knowledge which we possess of this substance. The pigment was discovered by MacMunn (2), who named it, according to the tissue in which it occurs, histohematin or myohematin; and he derived "modified histohematin" and "modified myohematin" which he distinguished from hemoglobin and its derivatives. Other investigators of the time did not admit the existence of histohematin as a separate pigment and it was a long time before its identity was verified, by Keilin. Keilin (3) proposed the name cytochrome as an expression of the universal distribution of the pigment, in aerobic cells, and he observed it, among the microorganisms, in yeast, and in *B. subtilis*. Yaoi and Tamiya (4) have made a survey of its occurrence among bacteria and find that the pigment is more abundant in the strict aerobes than in the facultative anaerobes and is wanting from the strict anaerobes. Cytochrome has, or more strictly, is defined by, a characteristic absorption spectrum. The four bands in the visible spectrum have their maxima at $m\mu$ 604, 566, 550, and 520, and are designated in order from red to violet, as *a*, *b*, *c*, and *d*. The *c* band is the most dense; *b* is often indistinctly separated from *c*, especially in the bacteria; the *d* band has three points of maximum absorption. The *a* band is the least distinct and appears to be lacking in many species of bacteria. These absorption bands are visible only when the pigment is reduced either by

* This work has been supported by a grant from the Chemical Foundation, Inc., New York City.

chemical agents or by the reducing processes of the cell. Cytochrome within the living cell may be seen to undergo an oxidation-reduction change.

Cytochrome has not been obtained in its entirety outside of the cell. MacMunn (5) obtained a typical two-banded hemochromogen (α 549, β 520, approximately) by extraction of muscle with ether. Keilin (3) obtained preparations of hemochromogen with similar spectra by extraction of yeast with water or with aqueous alkali. By treating yeast with strong KOH and allowing the preparation to stand 24 to 48 hours, Keilin obtained a clear solution with distinct bands at $m\mu$ 576, 552, and 524. The least modified preparation, according to Hill and Keilin (6), is represented by an aqueous extract which they called "cytochrome *c*." It shows bands at $m\mu$ 550 and 520 in the reduced form, and a faint diffuse band at 530 in the oxidized form. This preparation yields two porphyrins. One of these is identical with hematoporphyrin and may be converted into protoporphyrin. The other, obtained by a different procedure, has been described as different from any known porphyrin but appears to be very similar to coproporphyrin.

Schumm (7) has obtained by extraction with glacial acetic acid of yeast and a number of plant materials which contain cytochrome, "natural porphyratins," which are apparently identical with α -hematin, and yield protoporphyrin on disintegration. By extraction of yeast with alkali, Schumm has obtained an hemochromogen which appears identical with that of Hill and Keilin (6). Schumm found that the hemochromogen (the so called myochromogen) in similar preparations from muscle is converted by HCl into "myatin" which has the spectrum of α -hematin.

It has not yet been determined whether these acid and alkaline extracts of yeast each represent the corresponding form of a single substance as in the case of the hemochromogen and α -hematin from blood. Anson and Mirsky (8) have argued from the spectrochemical behavior of the hemochromogens of yeast when combined with pyridine and ammonia that they contain two different iron-porphyrin nuclei, one of which is identical with that of hemoglobin. Keilin (6) concluded from the spectrum of cytochrome *c* in pyridine, and its inability to combine with CO, that the iron-porphyrin portion of its molecule is different from ordinary α -hematin. The recovery of two different porphyrins from cytochrome *c*, as mentioned above, further distinguishes this hemochromogen from that derived from α -hematin.

Fischer and Schneller (9) obtained coproporphyrin as well as protoporphyrin from yeast. Fischer and Fink (10) have found coproporphyrin in old tuberculin (O.T.). Coproporphyrin is formed also by *C. diphtheriae* (1).

There is evidence therefore of the existence of different porphyrin nuclei in cytochrome.

EXPERIMENTAL

The microorganisms chosen for investigation were yeast, *B. phosphorescens*, and *C. diphtheriae*. These species grow vigorously and contain large amounts of cytochrome.

The initial material in the case of *B. phosphorescens* consisted of mass cultures, which were cultivated at room temperatures on the surface of sea water agar at pH 7.8. The bacterial mass was dried and ground *in vacuo* in a ball mill, then moistened with water, and subjected to alternate freezing and thawing. The pink opalescent solution, separated by the centrifuge from the undissolved particles, contains a hemochromogen pigment with strong absorption bands. The pigment is not autooxidizable. On oxidation with K_3FeCN_6 or H_2O_2 , the absorption bands disappear.

In the attempt to define this pigment by its electrochemical behavior, measurements of oxidation-reduction potentials were carried out on an extract obtained as described and brought to pH 7.6 with phosphate buffer. At the potential of +0.400 volt (referred to the hydrogen electrode) the absorption bands are not visible, but were seen to return during the negative drift, which follows addition of K_3FeCN_6 , when the potential had fallen to $Eh' + 0.345$. In consequence of the very sluggish response of the system to the oxidizing reagent and the presence of oxidizable substances other than the pigment, it is impossible to construct the oxidation curve of the pigment, but the Eo' value of the pigment may be fixed at approximately $Eh' + 0.280$ volt, if we assume that the pigment, in the concentration present, need have been only 10 per cent reduced in order for the bands to be faintly visible. In view of the special treatment, both chemical and theoretical, required in dealing with the oxidation-reduction potentials they will not be considered further here. It is our hope to be able to present more complete observations in a subsequent communication.

Although extraction by freezing and thawing must produce the least possible modification of the pigment, the method did not yield a sufficient amount of pigment for spectroscopic investigation in the case of microorganisms other than *B. phosphorescens*. Extractions with $N/1$ alkali, however, gave satisfactory yields, and NaOH extracts were obtained from *B. phosphorescens*, *C. diphtheriae*, and bakers' yeast. The preparations were deep orange or reddish in color, and were readily clarified by centrifugation.

The most suitable instrument for spectroscopic examination was found to be a spectrometer equipped with a grating of 10,100 lines per inch, and a single vertical cross hair. A ribbon filament lamp with a condenser was used for illumination. The solutions were placed for examination in rectangular cells.¹

¹ The cells supplied by Dargatz, Hamburg were found very convenient.

The alkaline extract of *B. phosphorescens* shows the hemochromogen-like spectrum, with bands about $m\mu$ 552 and 521. Hydrazine hydrate was added as a reducing agent, but the bands were visible before reduction. The axes of the bands have varied in different preparations from $m\mu$ 554 to 549 and from 522 to 519. These differences may be correlated with the degree of dispersion occasioned by the colloidal nature of the dissolved material, which is apparently largely protein, since after precipitation of dissolved protein by careful addition of acid the bands lie further to the violet. In a number of preparations containing relatively large amounts of pigment there has been present also a band of feeble intensity at $m\mu$ 575. On the addition of pyridine and hydrazine hydrate, no change in the position of the bands occurs. After adding KCN the bands are found at $m\mu$ 556 and 531. These observations indicate that the original extract did not contain α -hematin, since the main band did not fall in the position characteristic of pyridine hemochromogen ($m\mu$ 557.5) or of cyanhemochromogen ($m\mu$ 568.0).

The alkaline solution was treated with an excess of glacial acetic acid and extracted with ether. In nearly every case the pigment was precipitated along with protein by the action of the acid, but in a few experiments the ether extract showed two bands, one at $m\mu$ 575 and one between 539 and 535. This ether soluble pigment will be considered more fully below in cases where an apparently identical pigment was recovered in larger amount.

The alkaline extract of yeast is similar to that of *B. phosphorescens* in the number and position of the bands. Likewise after the addition of pyridine the bands are found in the original position; viz. $m\mu$ 576-572, 553-550, and 523-520.

The alkaline extract of *C. diphtheriae* shows a spectrum of the same type as that of *B. phosphorescens* and yeast. Certain bands however lie nearer the red end of the spectrum and have their axes as nearly as can be determined at $m\mu$ 556 and 526. The band about 575 is present, and in the majority of specimens an additional band has been seen at $m\mu$ 606-600. In this case also no shift in the bands takes place on the addition of pyridine.

The alkaline extracts are difficult to work with because of their high viscosity, and their large content of protein. Because of the small

yield, attempts to isolate the pigment from these solutions and from extracts obtained by freezing and thawing, without producing further modification of it, appeared unprofitable.

Extraction of the whole bacteria which had been freshly grown, washed with distilled water, and dried, was carried out with acetic acid and ether. The dried bacterial mass was moistened with water to form a paste and glacial acetic acid added, with constant stirring. Ether was then added and the mixture stirred. The ethereal layer was decanted and the procedure repeated with several fresh portions of ether.

Such acetic acid ether extracts of *C. diphtheriae* showed spectroscopically numerous bands, which were found to be due to two or more pigments. In the simpler cases the position of the bands was as follows:

m μ	622	574	535	about 500
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The two bands in the green were the densest. Extractions of such solutions with 5 per cent HCl separated a pigment which was identified as coproporphyrin, as follows:

Ether	I 623	III	526.3	IV 495.0
25 per cent HCl	I 592.5	III	549	
Cu compound in pyridine	564.8		528.2	

The ether residue after extraction with 5 per cent HCl showed only the bands at 575 and 539, and end absorption below 510. The substance responsible for these absorption bands appears to be identical with a porphyrin compound which is found in culture filtrates of *C. diphtheriae* and which has been described in a previous paper (1). Like that, this pigment in the extracts of the whole bacilli is broken down when an ethereal solution is shaken with 25 per cent HCl and yields coproporphyrin which appears in the aqueous acid phase. The same change takes place more slowly if the ethereal solution is evaporated to dryness, and the residue taken up in $\frac{M}{10}$ NaOH and allowed to stand for a few minutes. Concurrently with the degradation to coproporphyrin there appear in ethereal solutions faint bands at m μ 560.4 and about 525 which were found in the case of material

derived from culture filtrates to be due to the copper compound of coproporphyrin. In some cases a band at $m\mu$ 560.4 has been present in the original acetic acid-ether extracts of the whole bacilli.

Some preparations have shown, in addition to the bands already mentioned, bands due apparently to α -hematin. The band in the red has varied in position from $m\mu$ 644 to 636 depending apparently on the concentration of acetic acid in the ethereal solution. After shaking with 25 per cent HCl there remained in the ethereal solution a pigment which was identified spectroscopically as α -hematin as follows:

In pyridine and hydrazine hydrate

557.6 528.2

In $\frac{M}{10}$ NaOH and hydrazine hydrate

554.7 523.3

In acetic acid

644.1 544 505

The protoporphyrin liberated from the α -hematin, when combined with copper, gave the following values

In acetic acid-ether

569.1 531.7

In pyridine

575.5 538.2

Acetic acid-ether extracts of *B. phosphorescens* and yeast were similar qualitatively to those of *C. diphtheriae* and permitted identification of α -hematin, coproporphyrin, and the porphyrin compound described previously (1). The relative amounts of these substances, however, were different from those of *C. diphtheriae* extracts; α -hematin was apparently more abundant and in some preparations was the only pigment which could be detected. When present the bands of the porphyrin compound at $m\mu$ 575 and 535 were seen only in the original extract of the bacilli and after separation of the small amount of coproporphyrin could not be detected in the ether residue.

It became of interest to discover if the alkaline extracts which have been described would yield the same derivatives on extraction with acetic acid-ether as did the whole bacteria. An alkaline extract of *B. phosphorescens*, which showed bands at $m\mu$ 578, 550, and 522 when treated with acetic acid-ether yielded an ethereal solution with bands at $m\mu$ 575 and 539 and 502. The same result was obtained on repetition of the experiment, but the amount of pigment was too small to permit further investigation. An alkaline extract of *C. diptheriae* treated similarly, gave an ethereal solution with the same bands, and in addition a band at $m\mu$ 623 which was due probably to coproporphyrin, and a band at $m\mu$ 635 which was found to belong to α -hematin. Ethereal solutions prepared from alkaline extracts of yeast showed only the bands of α -hematin.

In the acetic acid-ether extracts of the whole bacteria were seen, in the blue portion of the spectrum, absorption bands which have not been previously mentioned. These were found to belong to lycopin, the red isomer of carotin. The bands, with Willstätter and Escher's (11) values* for comparison, were as follows:

In ether or petroleum ether

502 2	468.2	441 4
*510-449	480-468	440.

In carbon disulfide

549 2	502 2	
*554-540	514-499 5	479-472

A solution free from porphyrin was obtained by the method of Coward (2), by the use of petroleum ether; it showed the characteristic bands of lycopin. A chromatographic analysis, according to Tswett (13), for analyzing a mixture of carotinoid pigments was applied to a petroleum ether solution. Carotin, and xanthophyll a , a' , a'' , were not extracted from the bacilli as the characteristic color zones were not seen in the chromatograph. However, a narrow ring appeared at the top of the CaCO_3 column which gave evidence of xanthophyll b . The pigment was removed by washing with petroleum ether in absolute alcohol. The faintly colored solution was too weak for spectroscopic examination. Different portions of the

original pigment solution which passed through the CaCO_3 column gave the characteristic bands of lycopin which we had obtained in the acetic-ether extract.

A portion of the petroleum ether solution was evaporated to dryness and a few drops of concentrated H_2SO_4 were added. After standing for a few minutes a purplish blue color appeared. This reaction, however, is given by a number of organic compounds, *e.g.*, aromatic quinones, and cannot, therefore, be regarded as conclusive evidence in identifying carotinoids.

Although the study of carotinoids and related pigments in the non-chlorophyll bearing plants, *e.g.*, fungi, moulds, and bacteria, is particularly important in understanding their function in higher forms, we have not pursued the matter further in this investigation as our major interest has been the porphyrin pigments. Recognition of these pigments serves to prevent confusion between them and derivatives of cytochrome.

DISCUSSION AND SUMMARY

The pigment contained in the extracts obtained from *B. phosphorescens* by freezing and thawing, and in the alkaline extracts of *B. phosphorescens* and yeast, resembles the "cytochrome *c*" of Hill and Keilin (6) and the "porphyratin B" of Schumm (7) in giving absorption bands at $m\mu$ 552–550 and 522–520, but shows in addition a band about 575, as in the "hemochromogen A" obtained by Keilin (3) by prolonged treatment of yeast with strong alkali. Like cytochrome *c* the pigment of yeast extracts appears to be distinct from the ordinary hemochromogen of blood, because of the difference in position of the bands of the native materials and of the corresponding pyridine hemochromogens. On treatment with acetic acid, however, the yeast extract yields α -hematin, as identified spectroscopically. It is evident then that one portion of its iron-porphyrin nucleus is identical with α -hematin (iron-protoporphyrin), which must be present not as such, but in chemical combination.

The alkaline extracts of *C. diphtheriae*, compared with those of *B. phosphorescens* and yeast, show a constant difference in the position of the two bands in the green, which lie nearer the red end of the spectrum, at $m\mu$ 556 and 528. This extract likewise on treat-

ment with acetic acid yields α -hematin, which in the form of its alkaline hemochromogen may be responsible for the bands in the alkaline extract at $m\mu$ 556 and 528.

Great interest has attached in our investigation to the substance responsible for the absorption band in the alkaline extracts about 575. Extraction with acetic acid-ether of these alkaline solutions, as well as of the whole bacteria, yields a material which shows absorption bands at $m\mu$ 575-574 and 539-535, and appears to be identical with a complex porphyrin which has been found in culture filtrates of *C. diphtheriae*.

This complex porphyrin has been described in a previous paper (1). It is labile and breaks down readily to yield coproporphyrin and the copper compound of coproporphyrin, and is apparently the source of the coproporphyrin which is often found free in the culture filtrates. In the work repeated earlier we had been unable to obtain this complex porphyrin, or porphyrin compound, directly from the bacteria. In the present work we have been successful in obtaining it from the three species investigated.

The behavior of the complex porphyrin extracted from the whole bacteria is the same as of that found in filtrates. It is insoluble in 25 per cent HCl, and on disintegration gives coproporphyrin and the copper compound of coproporphyrin.

Information is quite lacking as to the particular form of combination in which this complex porphyrin occurs within the cell. The complex porphyrin is certainly not present there in the form in which it appears in the extracts. If diphtheria bacilli showing strong absorption bands of reduced cytochrome, while under examination with the microspectroscope are treated with glacial acetic acid, the bands of cytochrome are seen to fade and are replaced by those of the complex porphyrin at $m\mu$ 575 and 539.

The origin of the copper which is found, combined with coproporphyrin, as a product of the disintegration of the porphyrin compound, has been a matter of uncertainty. In the case of filtrates of *C. diphtheriae* it has seemed possible that the copper was never a constituent of the bacteria, and that combination with copper occurs only after the porphyrin has been liberated from the bacterial cell. With washed bacteria, however, the presence of copper in extracts indicates that

this element has been taken up from the culture medium and incorporated within the cell. Whether or not the copper is there combined with porphyrin cannot be decided by the present evidence. Copper occurs naturally, however, in combination with porphyrin in turacin (14), a pigment of the wing feathers of certain birds. In the present case such combination seems the more probable, so that the complex porphyrin may represent a form in which copper is contained within the cell.

Objection may be raised to the use of the term complex porphyrin or porphyrin compound for the substance referred to here and in the previous paper (1). The name hemochromogen might be applied with equal justification. Until the chemical nature of the substance is better known, however, it seems best not to use any but a simple descriptive name.

Reference should not be omitted here to the bacteriological significance of this compound, which arises from the correlation which we have previously observed between its amount and the content of toxin, in filtrates of *C. diphtheriae*. In respect to this porphyrin compound the pathogen *C. diphtheriae* seems to differ from the non-pathogenic forms in the readiness with which the material is liberated from the bacteria in cultures, rather than in the nature of the material.

CONCLUSIONS

1. Extraction of *B. phosphorescens* and yeast with alkali yields a hemochromogen similar to "cytochrome *c*" of Keilin. Preliminary measurements have been made of the oxidation-reduction potentials in aqueous extracts of *B. phosphorescens*.

2. Alkaline extracts of *C. diphtheriae* are similar to but are not identical with those of *B. phosphorescens* and yeast, with respect to the position of the main absorption bands.

3. From these extracts as well as from whole bacteria have been obtained by acetic acid-ether extraction, α -hematin, and a pigment apparently identical with the porphyrin compound previously described; this compound has a characteristic absorption spectrum, and on disintegration yields coproporphyrin and the copper compound of coproporphyrin.

4. This porphyrin compound which may be designated also a hemo-chromogen is a source of the coproporphyrin which may be extracted from bacteria. It may also represent a form in which copper is contained within the cell.

5. Lycopin, an isomer of carotin, has been isolated from *C. diphtheriae*.

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ELECTRIC PHASE ANGLE OF CELL MEMBRANES

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Höber (1910, 1912) was able to estimate the electric conductivity of the interior of red blood corpuscles from measurements made with high frequency alternating currents. Fricke (1924, 1925, *a, b*) greatly extended this work by careful analysis and measurements over a range of frequencies on the resistance and capacity of suspensions of these cells. He and McClendon (1926, *a, b*) both found the cell surface to behave like a rather good dielectric. It had an electric capacity that was practically independent of the frequency and a resistance so high as to be infinite within the limits of experimental error. But, in view of the highly specialized functions of these cells, it is not surprising to find that up to the present they seem to be the only biological systems showing these characteristics.

Measurements made at different frequencies on the alternating current impedances of various tissues by Philippon (1921), of muscle by Sapegno (1930), and of suspension of sea urchin eggs by Cole (1928, *b*) have indicated cell surfaces having capacities which vary with frequency. Measurements on the resistance and capacity of skin and nerve by Gildemeister and his students (1919-1928), Hōzawa (1925), Lullies (1928, 1930), and Krüger (1928), on *Valonia* by Blinks (1926) and on muscle by Fricke (1931) have further indicated a resistance which also varies with the frequency. In these latter measurements it has usually been found that the phase angle of this variable resistance-capacity combination remained more or less constant as the frequency was varied between wide limits. This resistance-capacity variation with constancy of phase angle is very similar to the phenomena of polarization capacity as found at metal-electrolyte interfaces.

Theory

In a previous paper (Cole, 1928, *a*) a theoretical relation was given between the alternating current reactance and resistance of an electric circuit composed of resistances and a single variable impedance element of constant phase angle. It is proposed to give a simple derivation of this result and to test it with the experimental data of others and our own which are now available.

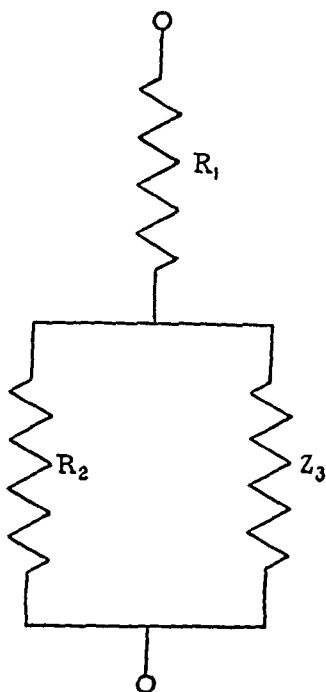


FIG. 1. Assumed equivalent tissue circuit

As was further stated, a system containing any number and arrangement of resistances with one and only one variable impedance element can be reduced to either one of two simple networks containing two resistances and one variable impedance element. For a specific example, the network of Fig. 1 will be considered. R_1 and R_2 are resistances while Z_3 is a complex impedance of resistance and reactance which will presently be restricted by the assumption of a constant phase angle. By the usual methods we have for the complex impedance of this circuit,

$$z = R_1 + \frac{R_2 z_2}{R_2 + z_2} = (R_1 + R_2) \frac{\frac{R_1 R_2}{R_1 + R_2} + z_2}{R_2 + z_2}.$$

For the applications which we shall make, z_2 will approach infinity at the low frequencies and will approach zero at the high frequencies, so we shall make the abbreviations

$$r_0 = R_1 + R_2, r_\infty = R_1, a = \frac{R_1 R_2}{R_1 + R_2}, a_1 = R_2. \quad (1)$$

We then have

$$z = r_0 \frac{a + z_2}{a_1 + z_2}.$$

We may now write $z = r + jx$ and $z_2 = r_2 + jx_2$ if we consider the resistance and reactance to be in series in each case. After separating the real and the imaginary parts of the resulting equation we have the two equations

$$\text{r.p. } (a_1 + r_2) r - x x_2 = (a + r_2) r_0,$$

$$\text{i.p. } (a_1 + r_2) x + r x_2 = x_2 r_0.$$

At this point let us assume a constant phase angle φ_2 for z_2 . Then $\tan \varphi_2 = x_2/r_2$ and we may write $r_2 = m x_2$ where $m = \cot \varphi_2$. Eliminating r_2 and x_2 ,

$$r^2 + x^2 - \left(r_0 + \frac{a}{a_1} r_0\right) r + m \left(r_0 - \frac{a}{a_1} r_0\right) x + \frac{a}{a_1} r_0^2 = 0.$$

And by equations (1) $\frac{a}{a_1} r_0 = r_\infty$ so

$$r^2 + x^2 - (r_0 + r_\infty) r + m (r_0 - r_\infty) x + r_0 r_\infty = 0.$$

This is however the equation of a circle in Cartesian coordinates where r and x are the variables. The radius is $\frac{r_0 - r_\infty}{2} \sqrt{1 + m^2}$ and the center is at the point $\frac{r_0 + r_\infty}{2}, -m \frac{r_0 - r_\infty}{2}$ so the slopes of the radii to r_0 and r_∞ are m and $-m$ respectively. It will be noticed that neither

the frequency nor the values of r_3 and x_3 enter explicitly into this circle diagram. It is a result of only the resistances r_0 and r_∞ and the phase factor m so all systems of the kind postulated having the same r_0 , r_∞ , and m will give the same circle irrespective of the distribution of the circuit elements. However, the frequency for which x is a maximum, *i.e.*, for the highest point on the circle, may be called a characteristic frequency of z_3 . It is the frequency for which the magnitude, but not the phase, of z_3 is equal to $r_0 - r_\infty$. Since x is treated as the coefficient of an imaginary quantity, this locus on the r, jx plane is called the complex plane representation.

In considering biological systems we shall have reactances due only to capacities so

$$x = -\frac{1}{c\omega} \quad \text{and} \quad x_3 = -\frac{1}{c_3\omega}$$

where c and c_3 are series capacities and ω is 2π times the frequency ν . In cases where the tissue has been balanced in a Wheatstone bridge by a resistance and a capacity in series, it is then only necessary to plot $1/c\omega$ *vs.* r . Where a parallel resistance-capacity combination has been used, the equivalent series circuit must be computed for each frequency. If a tissue has the equivalent of a single variable impedance element with a phase angle independent of frequency, then it must give a circle diagram. Any deviations from a circle must be due to the failure of this simple network as an electrical representation of the tissue.

Circle Diagrams

Fig. 2 is plotted from the data of Fricke and Morse (1925) on calf blood, after recomputation for the equivalent series resistance and reactance. The deviation of the points from the circle is not large in view of the difficulty of making such measurements at frequencies up to four and a half million cycles. It will be noticed that the center of the circle does not lie on the axis of resistances. Thus either m is not zero, as it would be for a perfect dielectric at z_3 , or the assumption of a single constant phase angle variable impedance element is not valid. This agrees with the impedance calculation of these data (Cole, 1928, *b*). From the diagram, $m = 0.16$.

Fig. 3 is a replotting of the data of Lullies (1930) on frog nerve. Lullies gave this identical representation, but apparently did not realize its significance. He computed m analytically from r_0 , r_∞ , r , and x but because of the behavior near r_∞ he came to the conclusion that m varied continuously with the frequency. Our analysis tends

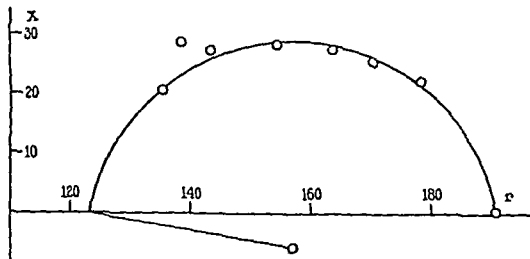


FIG. 2. Reactance vs. resistance in ohms for calf blood (Fricke and Morse)

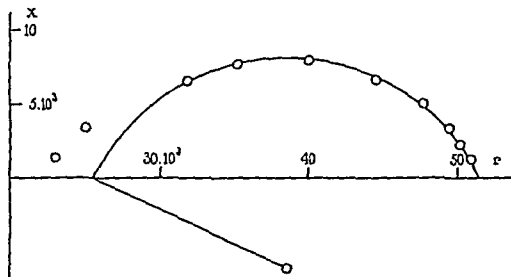


FIG. 3. Reactance vs. resistance in ohms for frog nerve (Lullies)

to show however that over the major portion of the frequency range the phase angle of a single variable impedance is sensibly constant, but with some other factor presumably entering at the highest frequencies. $m = 0.49$.

The data of Fricke (1931) on rabbit muscle have also been recom-

puted for the equivalent series combination and are shown graphically in Fig. 4. By an analytical method Fricke came to the conclusion that $m = 0.46$ which is an excellent agreement with the value obtained from the circle which best fits the data. The departure from the circle at the high frequencies is noticed here also. $m = 0.46$.

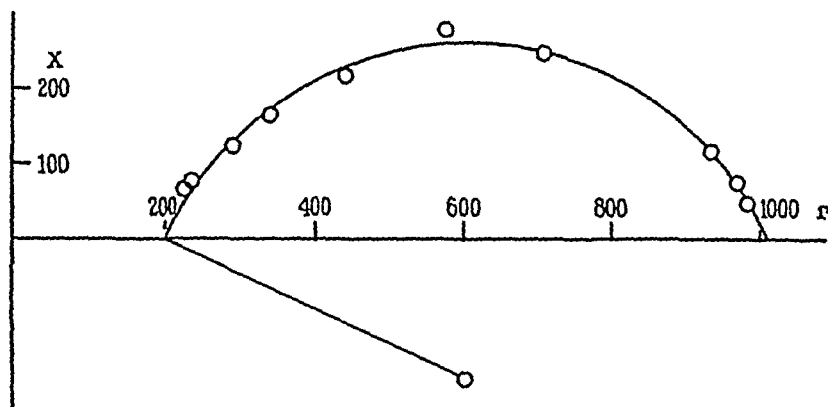


FIG. 4. Reactance *vs.* resistance in ohms for rabbit muscle (Fricke)

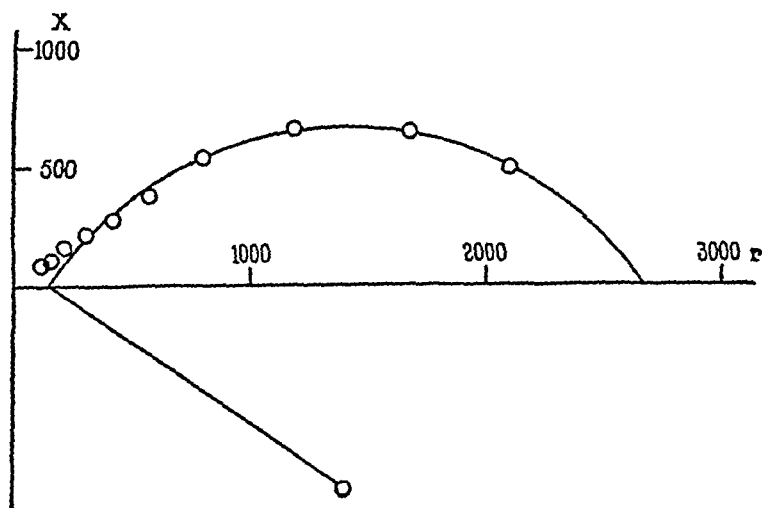


FIG. 5. Reactance *vs.* resistance in ohms for frog skin

Fig. 5 is the diagram for a double layer of frog skin in Ringer. The effective electrode area was 0.12 cm.^2 and the frequencies range from 100 to 90,000 cycles/sec. Once again we have a rather definite circle except at the high frequency end, and it should be noted that

no resemblance of a circle was obtained for low frequencies unless the bridge current was kept low. $m = 0.69$.

Since no physiological conclusions are to be drawn, the data on an excised cat diaphragm in Ringer shown in Fig. 6 are included. The tissue was irritable but the animal had been under ether for some time and the measurements were made at room temperature. The thickness was about 2 mm. and the effective electrode area was 0.28 cm.² The maximum frequency was not high enough to show any deviations which might be present in the high frequency portion of the circle. $m = 0.34$.

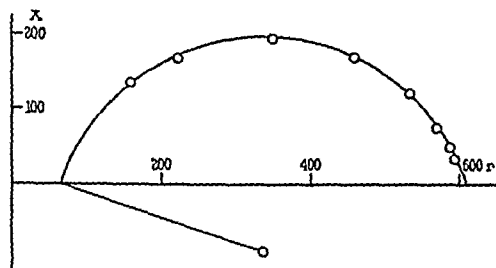


FIG. 6. Reactance vs. resistance in ohms for cat diaphragm

A slice of potato 2 mm. thick and 10 cm.² area in diluted sea water gave the data for Fig. 7. The sea water was diluted so that there was no appreciable change in the weight or the electrical properties. Again we cannot be sure that there would be no departure from the circle at higher frequencies. $m = 0.49$.

After a slice of potato had been boiled a few minutes in this solution, it had no measurable reactance and its specific resistance was approximately that of the solution.

The data for human skin do not extend to sufficiently low frequencies. Corresponding probably to the high frequency divergence of other materials, the measurements of Einthoven and Bijtel (1923), Gildemeister (1928), and Hōzawa (1932) give a straight line on the complex plane at frequencies above 1000 cycles. Hōzawa interprets

this as due to a static capacity in series with a polarization capacity which varies as the inverse square root of the frequency. At lower frequencies, the data indicate the possibility of an arc of a circle but Hōzawa's data to 250 cycles and our own to 50 cycles have not given a maximum for x .

On the other hand the highest frequency used in our experimental work, 90,000 cycles, was too low to give more than the initial portions of the corresponding diagrams for *Laminaria* and *Ulva*.

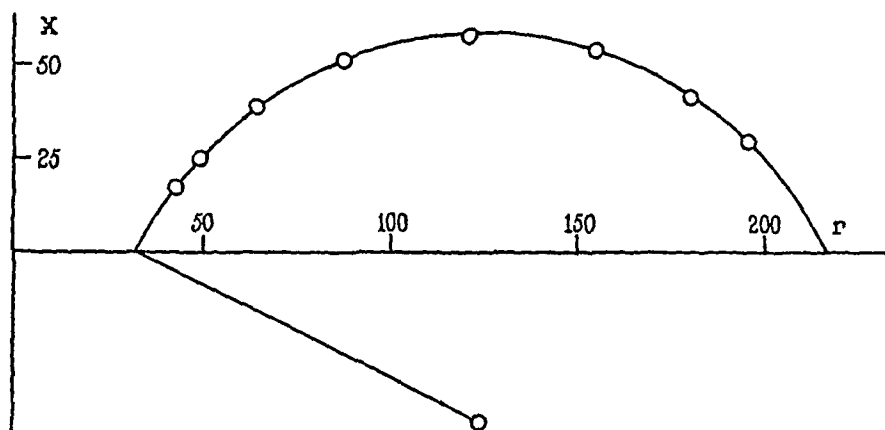


FIG. 7. Reactance *vs.* resistance in ohms for potato

DISCUSSION

In a system as complicated as tissue it might be supposed that there are at least as many variable impedance elements as there are cells. This would hardly seem to fit in with our assumption of a single element. In the less complex case of suspensions, it has been shown (Fricke, 1925, *a*; Cole, 1928, *a*) that all of the impedance elements are equivalent to a single impedance element. The evidence here presented tends to show that at least for low and intermediate frequencies, the tissues may be considered to have an equivalent single variable impedance element of constant phase angle.

The classical theory of a simple diffusion polarization capacity calls for $m = 1$ and $c_3 = \underline{c}_3 \omega^{-\frac{1}{2}}$ where \underline{c}_3 is a constant. For all of the above data however m is less than unity, but the values of c_3 have not been computed without the assumption of a constant m or a value of r_∞ . Fricke (1932) has shown theoretically that if $c_3 = \underline{c}_3 \omega^{-\alpha}$ where

α is a constant, then $1/\varphi_3 = \alpha\pi/2$ or $m = \tan \alpha\pi/2$. We may compute for each tissue the value of α from the value of m for the range in which m is constant.

SUMMARY

From the theory of an electric network containing any combination of resistances and a single variable impedance element having a constant phase angle independent of frequency, it is shown that the graph of the terminal series reactance against the resistance is an arc of a circle with the position of the center depending upon the phase angle of the variable element.

If it be assumed that biological systems are equivalent to such a network, the hypotheses are supported at low and intermediate frequencies by data on red blood cells, muscle, nerve, and potato. For some tissues there is a marked divergence from the circle at high frequencies, which is not interpreted.

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SOME PHYSICAL AND CHEMICAL PROPERTIES OF THE CELL SAP OF HALICYSTIS OVALIS (LYNGB.) ARESCH.

By G. J. HOLLENBERG

(From The Hopkins Marine Station, Pacific Grove)

(Accepted for publication, May 10, 1932)

During the summer of 1929, at The Hopkins Marine Station, at Pacific Grove, California, the following brief study was made of some of the physical and chemical properties of the cell sap of *Halicystis ovalis* (Lyngb.) Aresch. This was done in connection with a more extensive study by the writer on its morphology and reproduction which has, in part, been already reported by Dr. G. M. Smith (1930).

The specific gravity of the sap was determined by means of a small pycnometer drawn to a fine capillary for insertion through the cell wall of the plant. The latter was carefully cleaned and dried at the point of insertion and the sap of only one plant was used in each determination.

Determination of cell flotation was possible because occasionally plants could be severed from the *Lithothamnion* on which they grow without rupturing the cell wall at the point of attachment and without loss of turgor. These isolated cells sink readily in sea water in contrast to those of the Bermuda species (Blinks, 1927; 1929-30) which float. By varying the concentration of the solution in which they were immersed, a solution was obtained in which the plants were just suspended. It is interesting in this connection that the plant is able to withstand slight changes in the salinity of the water (0.46 to 0.55 molar) without apparent ill effects.

Barger's method was employed in determining vapor tension. This was by means of a capillary tube in which a small drop of the sap was placed between two similar drops of NaCl solution of a known concentration, the ends of the tube being sealed with oil. A concentration of the salt solution was finally found with which there was no change in the size of the drop of sap observable under a low power magnification with the use of an ocular micrometer. The method gives only approximate results.

In chloride determinations a small pycnometer was again employed to get a known volume of the sap from a single plant. Volhard's method of determination was used, adding excess AgNO_3 and titrating the excess with KCNS , using $\text{Fe}_2(\text{SO}_4)_3$ $(\text{NH}_4)_2\text{SO}_4$ as an indicator.

Sulfate was determined as BaSO_4 by means of a small weighing bottle with a fused glass filter after precipitation with excess BaCl_2 .

The pH was determined with brom cresol purple and brom cresol green.

The chief results are as follows:

Specific gravity (25°C.)

<i>Halicystis</i> cell sap (average of four samples).....	1.0257
Sea water at Pacific Grove (average of three samples).....	1.0258

Flotation (expressed as specific gravity) of total cell

Average of three vegetative plants.....	1.0265
Average of two plants in reproductive stages.....	1.0275

Vapor tension about equal to that of 0.60 M NaCl and practically identical with that of sea water at Pacific Grove (= 0.606 M*).

Chloride content

Average of three vegetative plants.....	1.926 per cent or 0.543 M
One plant recovering from a reproductive period	1.933 per cent or 0.545 M
Average of six plants in reproductive stages..	1.978 per cent or 0.558 M
Sea water at Pacific Grove (average of three samples).....	1.851 per cent or 0.523 M

Sulfate content

<i>Halicystis</i> sap (average of two samples of several plants each).....	0.013 per cent or 0.00135 M
Sea water at Pacific Grove (one sample) ..	0.279 per cent or 0.029 M

pH

<i>Halicystis</i> sap.....	5.4
Sea water at Pacific Grove.....	8.15*

Index of refraction

The N_d based on a number of samples ranged from 1.3384 to 1.3387 at 25°C. with little variation among different plants and practically identical with that of sea water at Pacific Grove throughout the range of temperatures studied (2.5–34.7°C.).

It may be added that Brooks (1930) has reported the $\text{K} \div \text{Na}$ ratio of the sap to be 1.5.

* This study was made under the direction of Dr. L. B. Baas-Becking at The Hopkins Marine Station. The figures marked with an asterisk were supplied by him.

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THE EFFECTS OF CO AND LIGHT ON THE OXYGEN CONSUMPTION AND ON THE PRODUCTION OF CO₂ BY GERMINATING SEEDS OF LUPINUS ALBUS

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(Accepted for publication, May 3, 1932)

I

When the rate of respiration of the germinating seeds of *Lupinus albus* was studied as a function of temperature, evidence was obtained suggesting that the mechanism governing the production of CO₂ may be quite different from that for the consumption of oxygen (Tang, 1930-31; 1931-32, *a*; 1931-32, *b*). A chemical test of this was deemed desirable.

The inhibiting effect of CO on the activities of organisms has been known since the time of Claude Bernard (1857). In the hands of Warburg (1926 *et seq.*) it was made to play an important rôle in the detection of the chemical agencies in respiration and fermentation. When yeast was subjected to various mixtures of CO and O₂, the oxygen consumption was found to be reversibly inhibited, while the production of CO₂ was not only not inhibited, but actually accelerated. It was further observed that the CO inhibition of oxygen uptake can be abolished by light, as in the case of the CO-hemoglobin combination accidentally discovered by Haldane and Smith (1896). Emerson (1926-27) employed this technic in the study of the oxygen consumption of the green alga *Chlorella*, where he found a photolabile CO inhibition when glucose is fed to the plants. Aside from these findings from Warburg's laboratory, the literature is singularly devoid of such observations with plants.

II

The seeds of *Lupinus albus* used were from the same lot as those used in the earlier studies, and were treated in exactly the same way (Tang, 1930-31); that is, they were soaked in distilled water for 12 hours and incubated on moist maple

sawdust (which previously had been repeatedly boiled) for 12 hours at $24 \pm 1^\circ\text{C}$. in darkness. At the end of this period the radicle has just penetrated the seed coat and is about 2 mm. long. The seeds were carefully picked up with forceps and mounted in the respirometer vessels.

Modified Warburg microrespirometers (Tang, 1931-32, *c*) were used in these experiments. The vessels are cylindrical in shape, and are of about 15 cc. capacity. They are attached to the manometers from the side instead of at the top. Each vessel has a side arm of the usual type with a ground-in glass stopper. The vessel proper is closed on the top with a ground-in glass stopper bearing a cross on its lower surface. On this cross the seed is fastened with the aid of a sulfur-free rubber band. Half a cc. of a 10 per cent KOH solution is placed in the vessel when oxygen consumption is to be followed, and 0.1 cc. of water, sufficient to maintain a moist atmosphere in the chamber, is substituted when CO_2 production is to be observed. The manometers are not shaken.

Carbon monoxide is prepared by dropping formic acid into hot concentrated H_2SO_4 , and is collected over water in a graduated bottle. Proper mixtures of CO and O_2 are obtained by introducing tanked oxygen into the bottle containing CO. For the routine series of experiments the volume proportion of CO to O_2 is as 76 to 24, approximating the oxygen content of the air in which the control experiments were carried out. The mixture is forced first over concentrated H_2SO_4 to remove water vapor (which often gives rise to serious errors in the manometer readings by condensing in the capillary of the limb connecting the manometer and the vessel), and then through the respirometers with the seeds already mounted in place. This is continued for at least 8 minutes, at the end of which the respirometers are closed to the atmosphere. When the production of CO_2 is to be followed, the respirometers are similarly filled with an atmosphere of N_2 or CO. The oxygen contents of the gas mixtures are checked by means of a Haldane gas analyzer. No attempt was made to purify the N_2 and CO from traces of oxygen, since preliminary experiments showed that no appreciable amount of oxygen was consumed in the unpurified gases.

In each series of experiments three respirometers were filled with CO or CO- O_2 mixtures and three with N_2 or air, depending upon whether the production of CO_2 or the consumption of oxygen was to be observed. All experiments were performed at 18°C ., in darkness, except as otherwise specified. The illumination, when needed, was from a 500 watt projection lamp 50 cm. above the vessels. The light traversed a layer of water in the thermostat about 5 cm. deep before reaching the seeds in the vessels. No change in temperature, either in the interior of the vessels or in the thermostat, was observed during the alternating periods of illumination and darkness. Just how much of the light reached the seeds, and how much of it was absorbed, was not determined, but an experiment to gain a rough idea of the depth of penetration of the light into the seed was performed in the following way. Arrangements were made to simulate the conditions of illumination of an actual experiment with the light source at a distance of 50 cm. from the seed, intercepted with a layer of water 5 cm. deep. The seed was sealed

with elastic cement to one end of a brass tube the other end of which was closely fitted to the window of a Macbeth illuminometer. The light which came through the seed was strong enough to match the light in the illuminometer when the scale of the latter was set at 20 millilamberts. Although a quantitative statement can not be made due to the difficulty in matching lights of different hues, it is quite obvious that under the specified conditions a considerable amount of light can penetrate into the center of the seed, as well as the hypocotyl, which is much more transparent than the seed itself.

The routine procedure for oxygen consumption measurements was as follows. Seven respirometers, with seeds mounted in six of them, were placed in the thermostat for $\frac{1}{2}$ hour to attain temperature equilibrium. Readings were then taken at 15 minute intervals for an hour, after which three of the respirometers were filled with CO-O_2 mixture. Another $\frac{1}{2}$ hour was allowed to pass and readings were resumed as before, in the dark, for an hour. Light was turned on and off at $\frac{1}{2}$ hour intervals thereafter until the end of the 3rd hour, when air was readmitted to the respirometers replacing the CO-O_2 mixture. After another $\frac{1}{2}$ hour of adaptation, the course of oxygen consumption was followed for an hour.

For CO_2 production, the procedures are essentially the same except that all the manometers save the one which was used as a thermobarometer were filled with N_2 at the beginning, and three of these, after the 1st hour, were refilled with CO . During the last hour, these were again filled with N_2 .

Since the seeds were germinated in the dark, no chlorophyll development occurred; hence during the short exposures to light photosynthesis and light *per se* were not complicating factors, as shown by the control experiment.

III

In all the experiments performed the following general picture results: the consumption of oxygen is markedly inhibited (19.6 per cent to 38 per cent) when the seeds are subjected to a 24 per cent O_2 -76 per cent CO mixture in the dark. When light is turned on, this inhibition is completely abolished, reappearing when the light is turned off. This can be repeated many times. The rapidity with which the effect appears varies from seed to seed. In most cases the response is evident in the first reading which is made 15 minutes after the light is turned on or off; in others, the response is delayed until the second reading. When returned to air, the inhibition disappears: in fact, not only does the inhibition disappear, but the rate of consumption of oxygen is considerably higher than what it had previously been in air.

For the production of CO_2 , there is little difference between the rate in N_2 and that in CO . In some of the cases there appears a slightly

accelerated rate of CO_2 production in CO , but the effect is too and too irregular to warrant a positive statement. Light effect on the rate of production of CO_2 either in N_2 or in CO . turning to N_2 : there is no acceleration of the rate as is the case consumption of oxygen; on the contrary, the rate appears lowered.

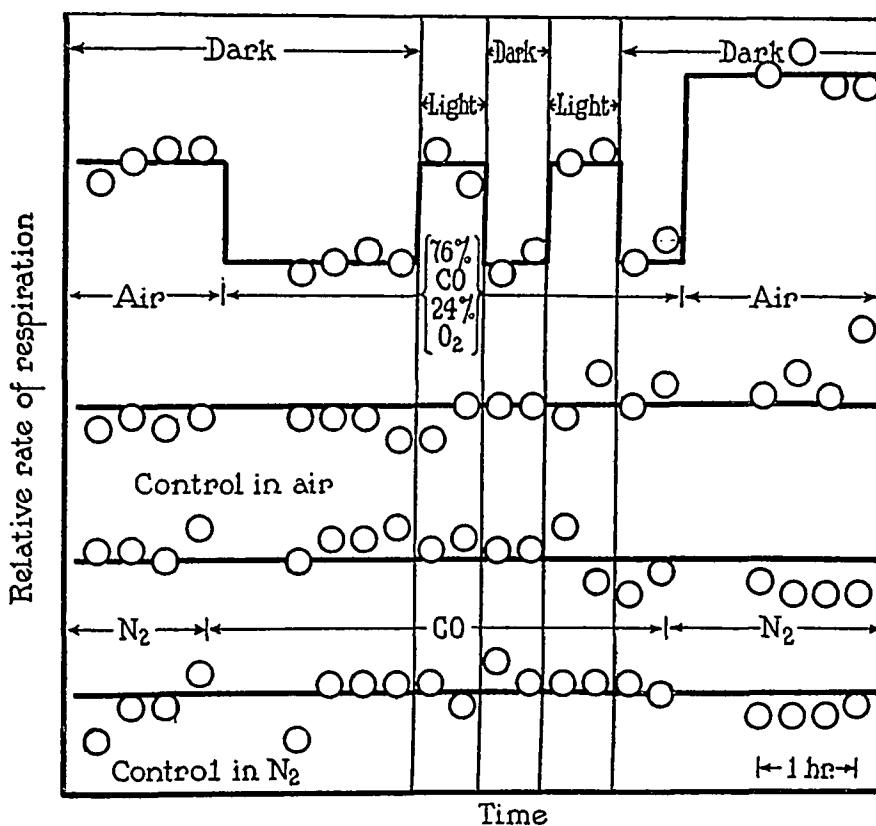


FIG. 1. Ordinate, relative rate of O_2 consumption or CO_2 production; ab time. Graphs 1 and 2 are for O_2 consumption, 3 and 4 are for CO_2 production and 4 are controls in air and N_2 respectively. Readings were taken at 15 min intervals. Alternating periods of the seeds in air, N_2 , CO , CO-O_2 mixtures and darkness, are indicated on the diagram.

These facts are semidiagrammatically represented in Fig. 1, complete protocols for which may be found at the end of this paper. Fig. 1, Graphs 1 and 2 are for oxygen consumption, and Graphs 3 and 4 are for the production of CO_2 ; 2 and 4 are controls in air and

respectively. The levels at which the four curves are drawn are purely arbitrary and are not related to the absolute magnitudes of the rates of gas exchange; only the course of the individual lines in air, CO, or N₂, in light or in darkness, is significant.

The data are presented quantitatively in Tables I and II. Table I presents the experiments dealing with (1) the rate of consumption of oxygen, (2) the degree of inhibition of this rate by CO, and (3) the extent of its acceleration on returning the seeds to air; (1) is expressed

TABLE I

Rates of O₂ consumption (Q_{O₂}) by germinating seeds of *Lupinus albus* in c.mm. O₂ per seed per hour at 18.0°C. in darkness. The seeds were first subjected to air, then to a mixture of 24 per cent O₂ and 76 per cent CO, and finally to air. The percentages of inhibition and acceleration are also presented.

Exp. No.	1			2			3		
	Q _{O₂}	Inhibi- tion	Accel- eration	Q _{O₂}	Inhibi- tion	Accel- eration	Q _{O₂}	Inhibi- tion	Accel- eration
		per cent	per cent		per cent	per cent		per cent	per cent
Air.....	69.0			60.0			63.0		
76 per cent CO, 24 per cent O ₂ ..	42.5	38.0		38.5	36.0		49.0	22.0	
Air.....	82.5		19.5	81.0		35.0	89.0		41.0
Exp. No.	4			5			6		
	Q _{O₂}	Inhibi- tion	Accel- eration	Q _{O₂}	Inhibi- tion	Accel- eration	Q _{O₂}	Inhibi- tion	Accel- eration
		per cent	per cent		per cent	per cent		per cent	per cent
Air.....	74.5			58.4			53.5		
76 per cent CO, 24 per cent O ₂ ..	49.0	34.0		44.7	23.4		43.0	19.6	
Air.....	100.0		25.0	75.0		28.4	90.5		68.0

in terms of c.mm. per seed per hour, (2) as a percentage $\left(\frac{Q_{O_2} - Q'_{O_2}}{Q_{O_2}} 100\right)$,

and (3) is expressed also as a percentage $\left(\frac{Q''_{O_2} - Q_{O_2}}{Q_{O_2}} 100\right)$. Q_{O₂}, Q'_{O₂,

Q''_{O₂ denote the rates of oxygen consumption per seed in air, in CO, and on return to air, respectively. Table II presents the rates of production of CO₂ in N₂ and CO.

A number of interesting observations were obtained besides those encountered in the routine experiments which may be recorded here. A series of experiments was made at a CO concentration of 83 per cent and an O₂ concentration of 17 per cent. The data are presented in Table III. It is noted that the percentage of inhibition is about 50 as compared to 19.6 to 38 when the proportion of CO to O₂ was as

TABLE II

Rates of production of CO₂ by the germinating seeds of *Lupinus albus* in N₂ and CO, at 18.0° C., in darkness; c.mm. CO₂ produced per seed per hour. The seeds were first subjected to an atmosphere of N₂, then to an atmosphere of CO, and finally again to N₂.

Exp. No.....	1	2	3	4	5	6
N ₂	63.2	38.6	42.2	43.5	48.5	44.2
CO.....	64.5	43.2	40.0	45.5	57.0	48.0
N ₂	51.8	33.4	40.0	38.0	40.5	36.0

TABLE III

Rates of O₂ consumption (Q_{O₂}) by germinating seeds of *Lupinus albus* in c.mm. O₂ per seed per hour at 18°C. in darkness. The seeds were first subjected to air, then to a mixture of 17 per cent O₂ and 83 per cent CO, and finally to air. The percentages of inhibition and acceleration are also presented.

Exp. No.....	1			2			3		
	Q _{O₂}	Inhibition	Acceleration	Q _{O₂}	Inhibition	Acceleration	Q _{O₂}	Inhibition	Acceleration
		per cent	per cent		per cent	per cent		per cent	per cent
Air.....	79.5			80.0			60.0		
83 per cent CO, 17 per cent O ₂ ..	37.5	48.0		40.0	50.0		28.0	54.0	
Air.....	90.0		13.3	92.5		15.8	59.5		0

76 to 24. Furthermore, when returned to air, the rates of oxygen consumption showed little or no sign of being accelerated. It is therefore evident that the degree of inhibition as well as the degree of subsequent acceleration of the rate of oxygen consumption on return to air are governed by the composition of the CO-O₂ mixtures. A comprehensive study of the effect of relative concentrations of CO and O₂ on the degree of inhibition was not attempted, mainly because the

problem would be complicated by the effect of oxygen concentration as such on the oxygen consumption by the seeds, and also by the fact that the rates of gas exchange in the individual seeds are not strictly comparable.

When the alternating periods of darkness and light are changed from $\frac{1}{2}$ hour to 15 minute duration each, the rates of consumption of oxygen in the CO-O_2 mixture for some seeds approach those under continuous illumination. In Fig. 2, Graph 1 is obtained from a seed kept first in darkness and then illuminated continuously for an hour. Graph 2

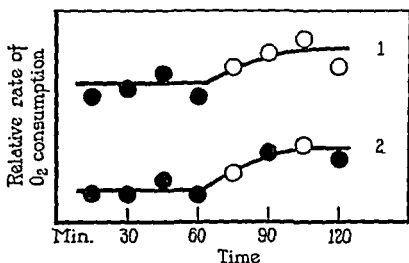


FIG. 2. Ordinate, relative rate of O_2 consumption; abscissa, time in min. Readings were taken at 15 minute intervals. In Graph 1 the seed was first subjected to darkness for an hour, and then was illuminated continuously for an hour. Graph 2 was obtained by subjecting the seed to darkness first, then to alternating periods of illumination and darkness of 15 minute duration each. Both were in a mixture of about 25 per cent O_2 and 75 per cent CO . Open circles denote readings for periods of illumination, solid ones denote those for dark periods.

2 is obtained from a seed kept first in darkness and then subjected to alternating periods of illumination and darkness of 15 minute duration each. Both were in a CO-O_2 mixture of about 25 per cent of O_2 and 75 per cent of CO , in darkness at 18°C . The open circles denote readings obtained for the periods in light, the solid circles are those obtained for periods of darkness.

When the seedlings with hypocotyls from 1 to 1.5 cm. long were placed in N_2 or CO , in darkness, at 22°C ., rough measurements did not reveal any elongation of the hypocotyls. When geotropically

excited, no sign of response was evident after 12 hours when the controls in air have long completed their reactions. These observations indicate that growth and geotropic curvatures are definitely related to the oxygen supply to the seedlings.

IV

From what appears in Table I, it may be stated that the degree of inhibition by CO of the oxygen consumption by the germinating seeds of *Lupinus albus* varies from seed to seed. This is not altogether unexpected, since the conditions of the seed coat may differ in different seeds in regard to thickness and extent of rupture, and since it is known that the rate of gas exchange differs among individuals. The important point is that in each case there is clearly an inhibition, which amounts to from 19.6 to 38 per cent. The acceleration of the rate after the return to air likewise varies from seed to seed, ranging from 19.5 to 68 per cent.

A survey of the data in Table II gives an indication of an accelerating effect of CO on the rate of production of CO₂ by the seeds: while this effect is slight and irregular, inhibition decidedly does not occur. When returned to N₂, after a period in CO, the seeds show a distinct decrease in the rate of the production of CO₂. These events are definitely different from those in the case of the consumption of oxygen.

This difference in the action of CO on the oxygen-consumption and on the production of CO₂ by the germinating seeds of *Lupinus albus* is what was expected from the differences in the temperature characteristics previously observed (Tang, 1930-31; 1931-32, *a*), and furnishes a chemical confirmation of the differences in the agents responsible for the two phases of respiration in *Lupinus albus* (Tang, 1931-32, *b*).

It is interesting to note that the rates of the production of CO₂ and of consumption of oxygen obtained in these experiments are of the same order of magnitude as those obtained previously, *viz.*, in the neighborhood of 60 c.mm. per seed per hour for the consumption of oxygen, and 45 c.mm. per seed per hour for the production of CO₂, with a respiratory quotient of about 0.75 at 18°. All these figures fall within the range of variation of those previously determined (Tang, 1931-32, *b*) at this temperature: possibly the figure for the rate

of production of CO_2 in N_2 is slightly lower than that in air, that is, the ratio of "intramolecular" CO_2 to normal CO_2 (I/N) is less than unity. Although the qualitative identity of this "anaerobic" CO_2 with that produced in air is lacking, it is hard to believe that the former is quantitatively replaced by the CO_2 produced by a different mechanism when oxygen is made accessible to the seeds. This point forms the subject of a subsequent investigation.

SUMMARY

The consumption of oxygen by germinating seeds of *Lupinus albus* can be reversibly inhibited by CO to a maximum extent of 36 per cent with a mixture of 24 per cent O_2 and 76 per cent CO at 18° , in darkness. This inhibition is completely abolished when the seed is illuminated. On returning to air, after a period in the CO- O_2 mixture, the rate of oxygen consumption is accelerated to as much as 68 per cent over what it had been previously, in air. The production of CO_2 is apparently not inhibited by CO. The bearing of these findings on the study of the rates of gas exchange of the seeds as a function of temperature is discussed.

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Protocol 1

Seeds soaked for 12 hours in distilled water, incubated on moist maple sawdust for 12 hours in darkness at $24 \pm 1^\circ\text{C}$. Experiments performed at 18°C . for oxygen consumption, in air or in a CO-O_2 mixture of 24 per cent O_2 , 76 per cent CO . Volume of seed, 0.6 cc. for Vessel 2, and 0.7 cc. for Vessel 1. Both contain 0.5 cc. 10 per cent KOH . First readings were taken $\frac{1}{2}$ hr. after placing of respirometers in thermostat.

Vessel 2: $K_{\text{O}_2} = 1.37$

Vessel 3: $K_{\text{O}_2} = 1.33$

$X_{\text{O}_2} = -\Delta h K_{\text{O}_2} = \text{c.mm. O}_2$

Time	$-\Delta h$ (mm.) in manometer*	
	No. 3 (control, in air)	No. 2 (first in air, then in CO-O_2 , finally in air)
11:30	0	0
45	13.5	12.5
12:00	14.0	13.5
15	13.5	14.0
30	14.0	14.0
1:15	0	0
30	14.0	8.5
45	14.0	9.0
2:00	14.0	9.5
15	13.0	8.5
30	13.0	14.0
45	14.5	12.5
3:00	14.5	8.5
15	14.5	9.5
30	14.0	13.5
45	16.0	14.0
4:00	14.5	9.0
15	15.5	10.0
5:00	0	0
15	15.0	17.5
30	16.0	18.5
45	15.0	17.0
6:00	18.0	17.0

* Figures in italics are those obtained in light.

Protocol 2

Seeds soaked for 12 hours in distilled water, incubated on moist maple sawdust for 12 hours in darkness at $24 \pm 1^\circ\text{C}$. Experiments performed at 18°C ., for CO_2 production in N_2 or CO . Volume of seeds, 0.6 cc.; volume of $\text{H}_2\text{O} \approx 0.1$ cc. in both cases. First readings taken $\frac{1}{2}$ hr. after the placing of respirometers in thermostat.

Vessel 1: $K_{\text{CO}_2} = 1.29$

Vessel 2: $K_{\text{CO}_2} = 1.42$

$X_{\text{CO}_2} = \Delta h K_{\text{CO}_2} = \text{c.mm. CO}_2$

Time	Δh (mm.) in manometer*	
	No. 1 (control, in N_2)	No. 2 (first in N_2 , then in CO , finally in N_2)
11:00	0	0
15	7.5	11.0
30	9.0	11.0
45	9.0	10.5
12:00	10.5	12.0
1:00	0	0
15	7.5	10.5
30	10.0	11.5
45	10.0	11.5
2:00	10.0	12.0
15	10.0	11.0
30	9.0	11.5
45	11.0	11.0
3:00	10.0	11.0
15	10.0	12.0
30	10.0	9.5
45	10.0	9.0
4:00	9.5	10.0
5:00	0	0
15	8.5	9.5
30	8.5	9.0
45	8.5	9.0
6:00	9.0	9.0

* Figures in italics are those obtained in light.

THE ACCUMULATION OF ELECTROLYTES

V. MODELS SHOWING ACCUMULATION AND A STEADY STATE

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(Accepted for publication, April 9, 1932)

Previous papers¹ point out the importance of the problem of accumulation in *Valonia*, and demonstrate that accumulation does not depend on the Donnan equilibrium or on the presence of ions or molecules inside which cannot pass out through the protoplasm. The experiments suggest that the following factors are important:

1. Permeability is determined by the properties of a continuous non-aqueous phase at both the inner and outer surface of the protoplasm.

2. Potassium and sodium penetrate chiefly as KOH and NaOH which may react with organic acids in the protoplasm to form salts which are decomposed by carbonic and other organic acids on reaching the sap.

3. The osmotic pressure of the sap is thereby raised and water enters.

4. This process tends toward a steady state where water and electrolyte enter at about the same rate, thus keeping the composition of the sap approximately constant as the cell grows.

5. Potassium penetrates more rapidly than sodium and in consequence potassium predominates over sodium in the sap.

6. Potassium accumulates inside because the sap is more acid than the external solution.

In order to see how far these suggestions form a workable scheme, it seemed desirable to test them by making a model.²

¹ Osterhout, W. J. V., (a) *Proc. Soc. Exp. Biol. and Med.*, 1930-31, 14, 285. (b) *J. Gen. Physiol.*, 1930-31, 14, 285. (c) *Biol. Rev.*, 1931, 6, 1. A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1930-31, 14, 285.

² The first models of this sort were made by the senior author. Reference was made to such models in a recent publication by Osterhout, W. J. V., and Stanley, W. M., *Proc. Soc. Exp. Biol. and Med.*, 1932, 29, 577.

We suppose that to enter *Valonia* electrolytes must traverse the outer non-aqueous layer of the protoplasm,³ then an aqueous layer, and finally an inner non-aqueous layer. As all the essential principles can be illustrated by one non-aqueous phase as well as by two, we use a model with a single non-aqueous layer (*B*, Fig. 1) placed between two aqueous phases, one of which, *C*, represents the sap; the other, *A*, corresponds to the solution bathing the outside of the living cell.

This model is a modification of the one employed by Irwin.⁴

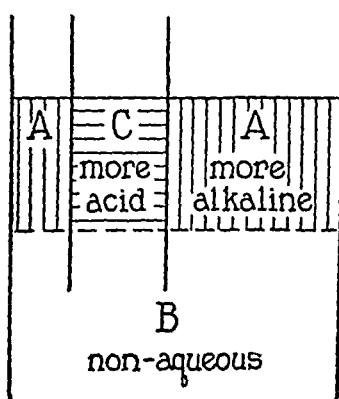


FIG. 1. Diagram of a model consisting of a non-aqueous layer, *B*, separating an aqueous layer, *C* (representing the cell sap), from an alkaline aqueous layer, *A* (representing the external solution).

A typical "large" model which will be called Model I was constituted as follows: About 900 cc. of the non-aqueous liquid was placed in the bottom of a battery jar (*B*, Fig. 2) 15 cm. in diameter and 22 cm. tall. Over this was placed about 2600 cc. of the alkaline solution so that the non-aqueous liquid formed a layer (*B*) about 5 cm. deep on the bottom of the jar, over which the aqueous alkaline solution formed a layer (*A*) about 15 cm. deep. A bell-shaped apparatus (*D*) was then lowered into the battery jar until its bottom edge was about 3 cm. below the surface of the non-aqueous liquid. This apparatus was prepared by sealing a piece of glass tubing, about 20 cm. long and having an internal diameter of 1.7 cm., to the bottom half of a 400 cc. beaker which had a hole of suitable size in the center of

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the bottom. This gave an apparatus about 25 cm. high, the lower portion of which consisted of a bell 4.5 cm. high and 8.5 cm. in diameter, which allowed the liquid in *B* and the liquid in *C* to have a fairly large surface of contact and yet permitted *C* to be of comparatively small volume. The alkaline liquid trapped in the bell when it was lowered was removed, and the interior of the bell flushed out with 100 cc. of distilled water by means of a pipette. Then the required solution was slowly added to the inside of the bell.

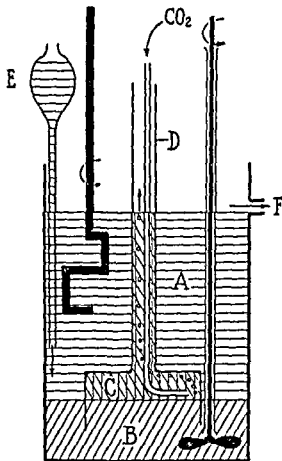


FIG. 2. Details of a model with a non-aqueous layer, *B*, separating two aqueous layers, *A* and *C*: all the layers are stirred.

When CO_2 was used as the source of the acid for *C* the bubbling stream of CO_2 from a tank was used as the stirrer for *C*: otherwise a glass stirrer was employed in *C*. The liquid in *A* was stirred by means of a bent glass rod. Layer *B* was stirred by means of a propeller placed so that the liquid was continually moved towards *C*. The shaft of the glass propeller for *B* was enclosed in a glass tube so that the interface between *B* and *A* was not disturbed.

In experiments where the alkaline solution *A* was continually renewed, the new solution was supplied from a separatory funnel *E* usually at the rate of 2 liters per day and the used solution was allowed to flow out the overflow outlet at *F*. After the steady state was reached the volume of *C* was maintained approximately con-

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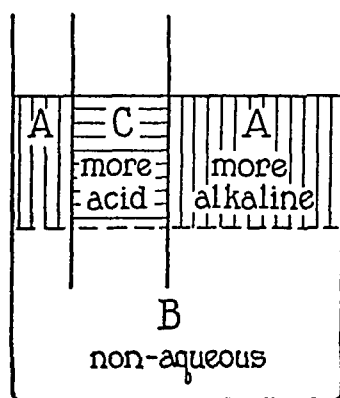


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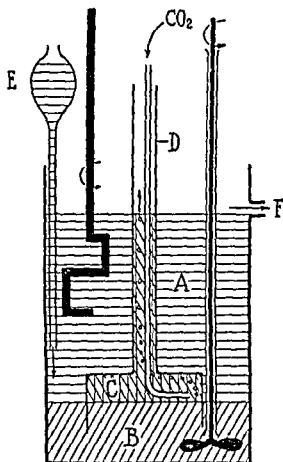


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In experiments where the alkaline solution *A* was continually renewed, the new solution was supplied from a separatory funnel *E* usually at the rate of 2 liters per day and the used solution was allowed to flow out the overflow outlet at *F*. After the steady state was reached the volume of *C* was maintained approximately con-

stant by the periodic removal of small amounts of liquid by means of a pipette. This was necessary because at the steady state the volume of *C* increases regularly. Thus the periodic removal of samples of *C* for analysis in no way depleted the volume of *C* in the model. The actual volume of *C* does not appear to affect the steady state, but it does affect the time required to reach the steady state, since the smaller the volume of liquid *C*; the more quickly is *C* affected by changes in *A*.

A smaller model, which will be called Model II, was prepared by using an ordinary 400 cc. Pyrex beaker to which an overflow outlet had been attached, as the outer container. The inner container consisted of a piece of large glass tubing 10 cm. long and 4 cm. in diameter, with its lower end 2 cm. below the top of the liquid in *B*, which formed a layer 5 cm. deep. The volume of solution in *C* was usually 20 cc. although this may be varied widely (10 to 45 cc.) by changing the height of the guaiacol-*p*-cresol layer, which also automatically regulates the volume of the solution in *A*.

A third model, which will be designated as Model III, is somewhat similar to Model I but the stirring is better and the surfaces between *A* and *B* and between *B* and *C* are much larger. The volume of the non-aqueous mixture has been greatly enlarged and the volume of *C* has been diminished. Hence potassium moves across about 25 times as rapidly as in Model I.

The temperature varied between 20° and 25°C.

Potassium was determined by the centrifuge method of Arrhenius,⁵ as used by Sherrill.⁶ This method was selected because of its great rapidity, as it was possible to make a fairly accurate potassium determination in less than 5 minutes. It is believed that this method is sufficiently accurate for comparative values, as in obtaining a time curve for increase of potassium concentration as in the experiments just described. The amount of unknown solution was adjusted so that the height of the precipitate in the centrifuge tube (Fig. 3) was always within about 20 per cent of that in the standard centrifuge tube with which it was compared. The standard used was 5 cc. of 0.5 *M* potassium which gave a precipitate upon centrifuging at about 1500 revolutions per minute for 3 minutes, varying very little from 2.6 cm. in height in the centrifuge tube. The concentration of potassium when the model had reached a steady state was usually checked by the perchlorate method. The potassium determinations made by the centrifuge method were accurate to within 5 per cent.

Sodium was determined by a centrifuge method which made use of the precipitate obtained with the magnesium uranyl acetate solution of Caley and Foulk.⁷ This precipitate was centrifuged in the tubes used for potassium and the height of the column was in each case compared with that given by a known amount of sodium. The amount of unknown solution was adjusted so that the height of the

⁵ Arrhenius, S., *Med. k. Ventenskapskad. Nobelinst.*, 1920, 4, No. 6, 1.

⁶ Sherrill, E., *Ind. and Eng. Chem.*, 1921, 13, 227.

⁷ Caley, E. R., and Foulk, C. W., *J. Am. Chem. Soc.*, 1929, 51, 1664.

precipitate in the centrifuge tube was always within about 20 per cent of that of the standard centrifuge tube with which it was compared. The standard used for sodium was 1 cc. of 0.0319 M sodium which gave a precipitate varying very little from 2.7 cm. in height. For example, if the unknown solution were known to contain somewhere near 0.05 M sodium, either by estimate based upon previous knowledge of the solution, or by means of a rough preliminary determination, then exactly 0.5 cc. of the unknown solution was placed in a centrifuge tube, made slightly acid to phenolphthalein with acetic acid (if already acid from mineral acids, these are neutralized with KOH and then made acid with acetic acid), and 10 cc. of the magnesium uranyl acetate added. The mixture was stirred for 30 minutes and then centrifuged at about 1500 revolutions per minute for 3 minutes. The concentration of sodium was then calculated from the height of this precipitate as compared with the standard. The sodium values so obtained were within 7 per cent of the sodium values obtained by the difference of the weights of the total chlorides or sulfates and the weight of KCl or K_2SO_4 , corresponding to the weight of potassium perchlorate found.



FIG. 3. Centrifuge tube for rapid determination of sodium and potassium consisting of a tube 3 to 6 cm. long with an internal diameter of about 1 mm. closed at one end and sealed to a piece of glass tubing 4 to 5 cm. long and about 2.4 cm. in diameter at the tip.

The determination of sodium bicarbonate in *A* was made by precipitation with Ba which was then converted to $BaSO_4$, and weighed as such.

Chlorides were determined by the Volhard method; sulfates as $BaSO_4$.

The authors desire to thank Dr. Marian Irwin for determining the pH values colorimetrically (using her modifications of the standard methods). These measurements were repeated by the authors using the same methods and the results were confirmed. To avoid loss of CO_2 from the liquid in *C* samples withdrawn in a pipette were delivered at the bottom of a test-tube beneath a layer of paraffin oil. The upper portion of the liquid in the pipette was rejected.

By using a comparator block³ for the liquid in *A* (colored by guaiacol) it was

³ The solution containing guaiacol was always placed toward the source of light in the comparator block. Comparison was made with the block in this position and the block was then reversed. The guaiacol solution, when turbid, can often be cleared by warming.

possible, with sufficient practice, to read differences of 0.1 pH in the region of 9.4, employing cresol phthalein⁹ as indicator. To ascertain whether guaiacol or *p*-cresol¹⁰ could affect the indicator (aside from an effect on the pH value) the following experiments were made. Since the solution used in *A* usually contained 0.04 M potassium (in the form of guaiacolate and of cresolate) + 0.01 M KHCO_3 a solution of double this concentration was made up and allowed to stand until the usual purplish color developed. It was found by the use of cresol phthalein to have a pH value of 9.3: we may call this X_1 . Buffer¹¹ of this pH value and of the same ionic strength (made so by the addition of KCl) was prepared: we may call this X_2 . Equal volumes of X_1 and X_2 were mixed to form X_3 . Presumably this involved little or no change in pH value. A colorimetric determination of X_3 (made immediately after adding thymol blue or cresol phthalein) gave a slightly higher pH value than for the pure buffer, X_2 (less than 0.1 pH higher in the case of borate buffer and less than 0.2 pH higher in the case of glycol buffer). Hence we may conclude that any disturbance, due to the action of guaiacol or *p*-cresol on the indicator may be neglected in the present case where only approximate values are needed. A repetition using one-half the concentration and employing cresol phthalein (the pH value being about 9.7) gave a similar result.

For the liquid in *C* similar experiments were made by using pure buffer and buffer saturated with guaiacol. Differences of 0.1 pH could easily be read by using cresol red. Here the concentration of guaiacol was much lower.

The buffers had in all cases about the same ionic strength as the samples and were carefully checked by means of the hydrogen electrode.¹² The indicators (freshly made up) were sufficiently close to isohydric to avoid disturbing effects. Care was taken to make prompt readings before any change in color occurred.

There is a little variation in the pH value of *A* in the steady state, due to slight variations in the rate of flow of the external solution and variations in the rate at which CO_2 or bicarbonate diffuses in from *C* (the amount picked up from the air is negligible).

⁹ Especial care was devoted to selecting test-tubes of uniform size and to their cleanliness. Light from a daylight lamp was reflected from a metal surface painted with white lead and was brought to the optimum intensity. The comparator block was screened on all sides so that no extraneous glare disturbed the operator. The amount of indicator was reduced as far as was compatible with good reading.

¹⁰ A saturated solution of *p*-cresol in water at 20°C. is about 0.2 M and that of guaiacol about 0.14 M.

¹¹ Both borate and glycol buffers were used. Regarding borate buffers see: Michaelis, L., *J. Biol. Chem.*, 1930, **87**, 33.

¹² In the case of the hydrogen and glass electrodes there is some uncertainty as to the need for correction of liquid junction potentials at higher ionic strengths and at high and low pH values (*cf.* MacInnes, D. A., and Belcher, D., *J. Am. Chem. Soc.*, 1931, **53**, 3315; also Clark, W. M., *The determination of hydrogen ions*, The Williams & Wilkins Co., Baltimore, 3rd edition, 1928).

In a preliminary experiment with Model I, using 2000 cc. of 0.03 M KOH in *A* and 100 cc. of 1.0 M HCl in *C*, there was an accumulation of KCl in *C* amounting in the course of 115 hours to 0.43 M . The concentration in *A* fell off so that the ratio of potassium in *A* and *C* was $0.43 \div 0.0019 = 226$ or an accumulation of potassium in *C* of over 200 times that in *A*.

At the end of 67 hours *A* was found to be neutral (due to the outward passage of HCl) and to contain 0.008 M potassium and 0.007 M chloride, so *A* was removed and replaced by a 0.008 M KOH solution (at this time there was 0.008 M potassium outside and 0.36 M potassium inside so that there was a marked accumulation). This was found to be necessary again at the end of 98 hours when *A* was removed and replaced by a 0.0035 M KOH solution. At the end of 115 hours the concentration of potassium in *A* had fallen from 0.03 M to 0.0019 M due to the passage of 56.9 millimols of potassium from *A* into *C*. During this same period of time 21 millimols of HCl passed from *C* into *A*. *B* consisted of 70 per cent guaiacol + 30 per cent *p*-cresol.

Most of the experiments were made by using CO_2 as the acid inside. As an example of the results we may consider an experiment in which the non-aqueous layer (*B*) consisted of 70 per cent guaiacol and 30 per cent *p*-cresol; at the start the aqueous solution in *A* contained 0.05 M KOH and *C* consisted of distilled water; CO_2 was continually bubbled through *C* during the entire experiment.

At the end of 50 hours the concentration of potassium in *C* was more than 5 times as great as in *A* so that it was evident that a marked accumulation was possible.¹³ Practically all of this was in the form of $KHCO_3$. A certain amount of guaiacol and *p*-cresol dissolved¹⁰ in *C* and much of the KOH in *A* was converted to salts of guaiacol and *p*-cresol.

It seemed desirable to keep the solution in *A* approximately constant. This could be done by allowing KOH to flow in a steady stream through *A*. But as this would remove some of the non-aqueous liquid in *B* it was decided to shake the KOH with the mixture of guaiacol and *p*-cresol before allowing it to enter *A*. This, of course, converts most of the KOH to K-guaiacolate and K-*p*-cresolate but

¹³ Accumulation also occurred when formic or citric acid was placed in *C*. Both of these acids passed through *B* to *A* very freely, consequently it was necessary to add formic and citric acid to *C* daily.

the principle remains unchanged since in any case these salts must be formed and it makes no difference whether they are formed before or after entering *A*: hence we may continue to speak of the penetration of KOH into *B* and *C* even though it be shaken with the liquid taken from *B* before entering *A*.

A new experiment was started in which 0.05 *M* KOH was shaken

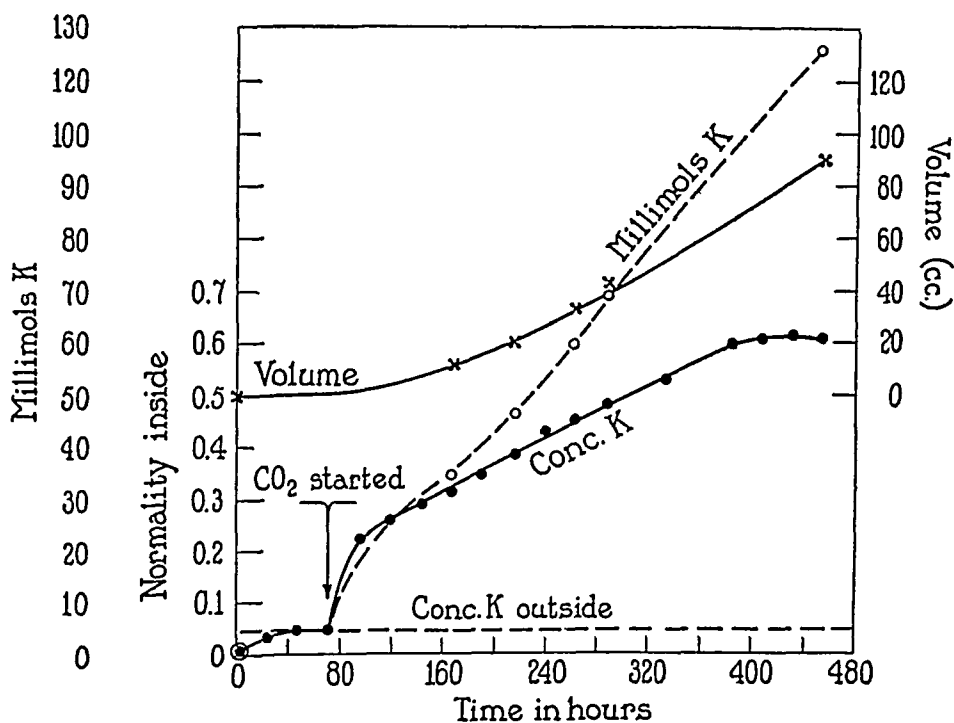


FIG. 4. Changes in the "artificial sap" in an experiment (No. 65, Table I) with 0.05 *M* KOH (previously shaken with some of the non-aqueous mixture of 70 per cent guaiacol and 30 per cent *p*-cresol taken from *B*) in *A* and distilled water in *C*. There was no accumulation until the CO₂ began to bubble in *C*.

with a guaiacol-*p*-cresol mixture¹⁴ for 2 minutes, allowed to separate, and then made to flow in a steady stream through *A*.¹⁵ Under these

¹⁴ The amount of this was so small that it took up only a negligible amount of potassium.

¹⁵ This is essentially the same as placing 0.05 *M* KOH in *A* and allowing the organic salts to form at the surface of *B* and then pass into *B*. As fast as potassium disappears from *A* it may be replaced by the addition of fresh KOH (or a steady stream of KOH may be allowed to flow through *A*: but this carries off more of the non-aqueous mixture).

circumstances the flowing liquid in *A* (containing about 0.05 M potassium combined with guaiacol and *p*-cresol) presumably dissolved little or none of the guaiacol or *p*-cresol in *B* but salts of potassium and sodium passed into *B* and on reaching *C* (where CO₂ was bubbling) were decomposed to form bicarbonates¹⁶ and carbonates.

During the first 71 hours no CO₂ was bubbled through *C*, and potassium entered until its concentration was equal to that in the external solution (0.05 M). There was no increase in the volume of liquid in *C* (Fig. 4).

The bubbling of CO₂ was then commenced and in the course of 312 hours the concentration¹⁷ of potassium in *C* rose to 0.61 or 12 times that in the external solution (Exp. 65, Table I). From this time on water was taken up as rapidly as electrolyte¹⁸ so that although potassium continued to enter (as shown by the curve labelled "Millimols K," Fig. 4) the concentration in *C* remained approximately constant. This is presumably analogous to what happens when *Valonia* takes up water and grows while the concentrations in the sap remain approximately constant.

This was repeated with Model II which had larger specific surfaces so that the speed of penetration was greatly increased. The ratios of the volumes, however, remained as before¹⁹ and the steady state was reached with 0.60 M potassium inside (Exp. 73, Table I). With Model III somewhat higher values were attained (Table I).

Once established, the steady state is quite stable, responding but slowly to changes in concentration in *A* or to changes in the rate of bubbling CO₂ in *C*. In this respect it may resemble certain living cells.

A rather extreme test of the stability of the steady state is shown in

¹⁶ With no CO₂ bubbling through *C* the entrance of potassium was very slow and no accumulation occurred. This is to be expected since as soon as the concentration of potassium in *C* became equal to that in *A* the pH values would be approximately equal.

¹⁷ This was mostly KHCO₃.

¹⁸ This evidently depends on the fact that the osmotic pressure inside increases as the concentration of KHCO₃ and NaHCO₃ increases.

¹⁹ The external solution in all these cases contained 0.05 M potassium in the form of guaiacolate except for 0.008 M to 0.012 M KHCO₃ (due to CO₂ diffusing from *C* through *B* into *A* and varying with the rate of flow of the liquid from *E*, Fig. 2).

TABLE I
Steady State
 (When *B* Consists of 70 Per Cent Guaiacol + 30 Per Cent *p*-Cresol)

Model	Ex-periment	Outside (A)		Inside (C)		Activity coefficient of K		$\frac{\Delta F}{2.3 RT}$ for KOH	Hrs. required to reach steady state
		Composition	pH	Composition	pH	Out-side	In-side		
II	74	0.04 K-org.* 0.01 KHCO ₃ †	9.1	0.63 KHCO ₃	7.5	0.817	0.63	0.61	—
III	68	0.033 K-org. 0.017 KHCO ₃	9.1	0.75 KHCO ₃	7.5	0.817	0.618	0.55	18
III	69	0.033 K-org. 0.017 KHCO ₃	9.1	0.83 KHCO ₃	7.5	0.817	0.612	0.51	18
I	65	0.04 K-org. 0.01 KHCO ₃		0.61 KHCO ₃					312
II	73	0.04 K-org. 0.01 KHCO ₃		0.60 KHCO ₃					25
I	66	0.043 K-org. 0.007 KHCO ₃ 0.043 Na-org. 0.007 NaHCO ₃	9.4	0.73 KHCO ₃ 0.45 NaHCO ₃	7.5	0.767	0.587	1.11	367
III	78	0.011 K-org. 0.014 KHCO ₃	8.9	0.34 KHCO ₃	7.3	0.848	0.670	0.57	60
III	79	0.011 K-org. 0.014 KHCO ₃	8.8	0.32 KHCO ₃	7.2	0.848	0.675	0.59	52
I	64	0.041 K-org. 0.009 KHCO ₃	9.1	0.63 KHCO ₃	7.6	0.817	0.63	0.60	208

* K-org. means potassium combined with guaiacol and *p*-cresol.

† The bicarbonate in *A* was derived from CO₂ or HCO₃⁻ diffusing in from *C*.

Fig. 5. The steady state was reached²⁰ at 208 hours with 0.63 M KHCO₃ inside (Exp. 64, Table I), and at 259 hours enough KHCO₃

²⁰ In this experiment the external solution was 0.05 M KOH which had been shaken with liquid from *B* (as in the previous experiment): CO₂ was bubbled in *C*.

was added to *C* to make the concentration 1.1 *M*. After this potassium continued to enter as shown by the curve labelled "Millimols K" but water entered so rapidly (as shown by the curve labelled "Volume") that the concentration of potassium approached its former

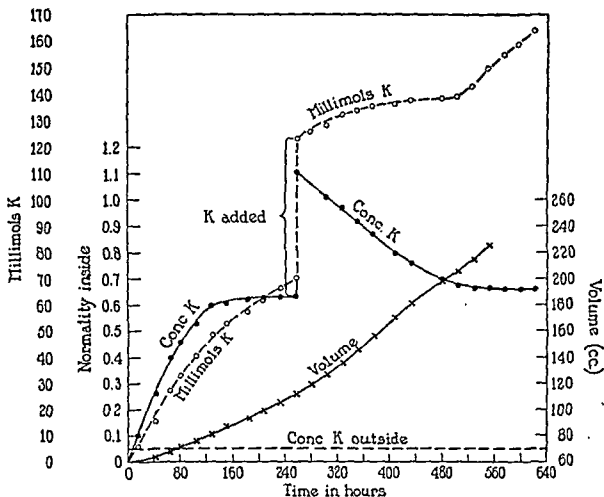


FIG. 5. Changes in the "artificial sap" in an experiment (No. 64, Table I) with 0.05 *M* KOH (previously shaken with some of the non-aqueous mixture of 70 per cent guaiacol and 30 per cent *p*-cresol taken from *B*) in *A* and distilled water plus CO₂ in *C*. After the steady state was reached enough KHCO₃ was added to *C* to raise the concentration of KHCO₃ from 0.63 *M* to 1.1 *M*. After this potassium continued to enter but as water passed in more rapidly (due to the increase in osmotic pressure in *C*) the concentration of potassium in *C* fell off almost to the previous level of the steady state.

level. It is evident that the steady state can be approached from either side.

The steady state is attained more rapidly when the temperature is raised. The diffusion of all substances is accelerated but the

transfer of water is hastened more than that of salt so that the steady state is reached at a lower salt concentration.

One of the most striking facts about *Valonia* is the selection of potassium in preference to sodium. To see whether the model would imitate this the experiment just described was repeated with 0.05 M KOH + 0.05 M NaOH in *A* in place of 0.05 M KOH. The results are

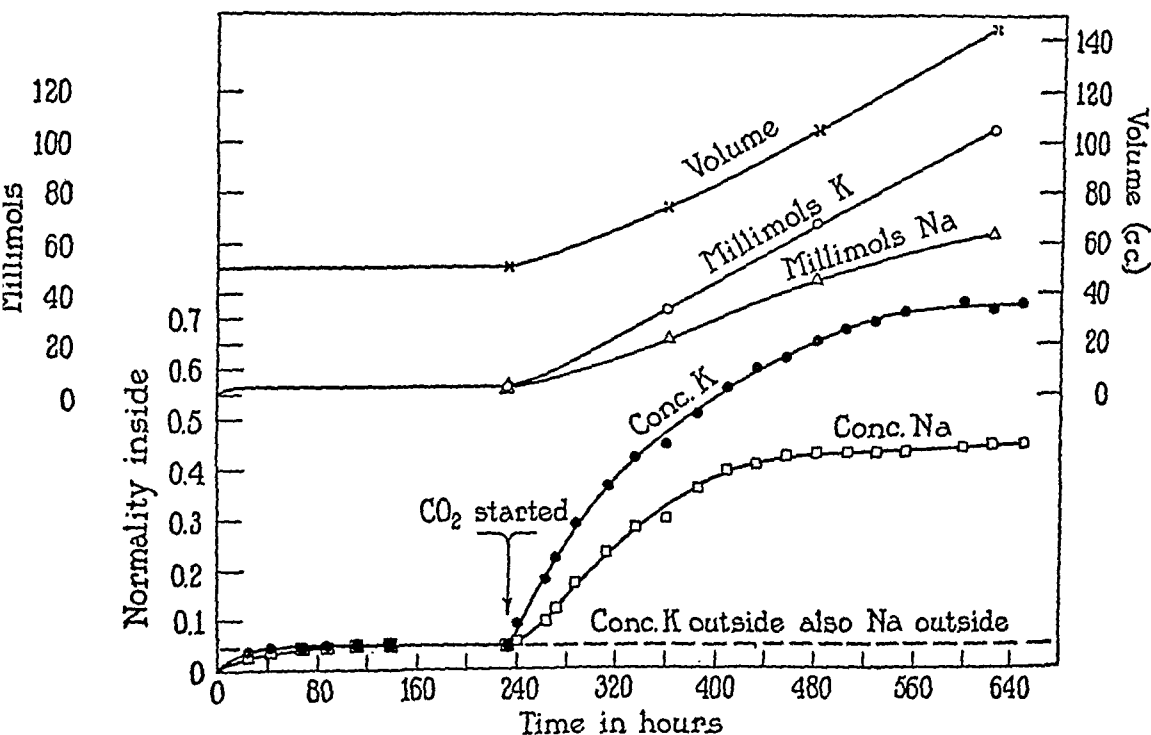


FIG. 6. Changes in the "artificial sap" in an experiment (No. 66, Table I) with 0.05 M KOH + 0.05 M NaOH (previously shaken with some of the non-aqueous mixture of 70 per cent guaiacol + 30 per cent *p*-cresol taken from *B*) in *A* and distilled water plus CO₂ in *C*. The ratio of potassium to sodium in the steady state is about 8 to 5.

shown in Fig. 6. At the end of 367 hours after starting the CO₂ the concentration of potassium was 0.71 M (*i.e.* about 14 times that in *A*) and the concentration of sodium was 0.43 M (*i.e.* about 8.6 times that in *A*). From this time on water entered about as rapidly as electrolyte, *i.e.* a steady state occurred which behaved like the steady states already discussed. (Exp. 66, Table I.)

Variations of the non-aqueous mixture can change these relations. On increasing the proportion of *p*-cresol the $K \div Na$ ratio falls off. A preponderance of sodium over potassium is found with (a) 100 cc. cyclohexanol + 2 gm. palmitic acid, (b) 50 cc. amyl alcohol + 4 gm. stearic or palmitic acid.

It would appear that in certain respects the model follows rather closely the hypothesis outlined in previous papers²¹ to explain the behavior of *Valonia*. Attention may be called to the following:

1. KOH tends to enter because the external product (K_o) (OH_o) is greater²² than the internal product (K_i) (OH_i) (where the subscripts *o* and *i* denote activities outside and inside respectively).

2. Potassium predominates over sodium in *C*²³ (i.e., in the "artificial sap"), because the non-aqueous layer is more permeable to certain K-compounds.

3. KOH combines with carriers in the non-aqueous phase to form organic salts which react with the weak organic acid in the "artificial sap."

4. The salts thus formed in the "artificial sap" raise its osmotic pressure above that of the external solution and in consequence water enters. In the model this occurs without restraint but in *Valonia* the cell wall can inhibit the entrance of water more or less completely.²⁴

In Exp. 66 (Table I) the formation of bicarbonates of potassium and sodium raised the osmotic pressure in *C* from nearly zero to more than 40 atmospheres (more than 10 times that of the external solution). If CO_2 act thus in the living cell (as elsewhere suggested²⁵) it plays an important rôle in the creation of osmotic energy in the cell.

5. Equilibrium is not attained but a steady state is reached in which the entrance of water keeps pace with that of electrolytes so that the

²¹ Osterhout, W. J. V., *J. Gen. Physiol.*, 1930-31, 14, 277; *Biol. Rev.*, 1931, 6, 369.

²² Unless the internal pH value be lower than the external there is no accumulation of potassium.

²³ The ratio $K \div Na$ is much greater in *Valonia* than in the model.

²⁴ The rate of increase in volume in *C* with Model I was in the neighborhood of 10 per cent per day or more than 10 times that of *Valonia* under favorable laboratory conditions. The rate of stirring of the solutions and the extent of their surface are determining factors in the model.

²⁵ Osterhout, W. J. V., *Proc. Soc. Exp. Biol. and Med.*, 1926-27, 24, 234; *J. Gen. Physiol.*, 1930-31, 14, 285; *Biol. Rev.*, 1931, 6, 369.

composition of the sap remains approximately constant. This is presumably analogous to what is found in *Valonia*. In this steady state the difference between internal and external pH value is about 2, both in the model and in *Valonia*.

In discussing the penetration of KOH into *Valonia* it has been stated elsewhere²⁵ that this takes place as long as the external product $(K_o)(OH_o)$ is greater than the internal product $(K_i)(OH_i)$. In other words, it continues as long as there is a difference between the thermodynamic potential of KOH inside and outside. We may call this difference ΔF and write²⁶

$$\Delta F = 2.3 RT \log \frac{(K_o)(OH_o)}{(K_i)(OH_i)}$$

Since the ionic strength is nearly the same inside and outside we may substitute concentrations for activities and write

$$\Delta F = 2.3 RT \log \frac{(0.012) 10^{-6}}{(0.5) 10^{-8.2}} = 2.3 RT (0.58)$$

In the model KOH never reaches a condition of equilibrium in which $(K_o)(OH_o) = (K_i)(OH_i)$ but instead attains a steady state, where ΔF is not very different from what it is in *Valonia*. Since the ionic strength is not the same inside and outside we endeavor to approximate the activities. Probably the most reasonable way to do this, in the absence of actual determinations, is as follows: In one experiment (Table I, Exp. 74) the outside solution had in the steady state a pH value of 9.1 (*i.e.* $OH_o = 10^{-4.9}$) and contained 0.05 M potassium (in the form of guaiacolate and bicarbonate²⁷). For purposes of calculation we take the activity coefficient of potassium to be that of potassium in a solution of KCl of the same ionic strength, in which the activities of potassium and chloride are regarded as equal.²⁸ The activity

²⁵ This results from the equations $F_{outside} = F_o + 2.3 RT \log(K_o)(OH_o)$ and $F_{inside} = F_o + 2.3 RT \log(K_i)(OH_i)$, where F is the thermodynamic potential and F_o is the value of F for 1 mol of KOH in the standard state. Cf. Lewis, G. N., and Randall, M., *Thermodynamics*, New York, McGraw-Hill Book Co., Inc., 1923.

²⁷ CO₂ diffuses from C into A as the solution flows through A.

²⁸ MacInnes, D. A., *J. Am. Chem. Soc.*, 1919, 41, 1086. Cf. Güntelberg, E., and Schiödt, E., *Z. phys. Chem.*, 1928, 135, 393.

coefficients thus obtained are by no means exact but they will at least give us an approximation sufficient for our present calculation. We then have

$$\begin{aligned}\Delta F &= 2.3 RT \log \frac{(K_o) (OH_o)}{(K_i) (OH_i)} \\ &= 2.3 RT \log \frac{(0.05) (0.817) 10^{-4.9}}{(0.63) (0.63) 10^{-6.4}} \\ &= 2.3 RT (0.61)\end{aligned}$$

From this we see that if the pH value of *Valonia* sap were 5.5 (giving $OH_i = 10^{-8.5}$) the value of ΔF would be the same as in the model. Such a low value frequently occurs. We have used 5.8 in the equation but this may be a little too high to represent the usual value. In some experiments the values of ΔF were greater than this but in others less than in *Valonia* (Table I).

6. The thermodynamic potential of the potassium salt inside becomes greater than outside so that $(K_i) (HCO_{3,i}) > (K_o) (HCO_{3,o})$. In *Valonia* the potassium salt inside is KCl and we find $(K_i) (Cl_i) > (K_o) (Cl_o)$.

7. The steady state in our models and in *Valonia* differs from the Donnan equilibrium as is evident from Table II which shows some typical cases. (In the Donnan equilibrium all the ratios in each horizontal row should be equal.)

8. Does accumulation depend on the presence of ions which are unable to pass through the non-aqueous layer? Evidently not, for all the ions in the system can penetrate freely (this expression is used regardless of whether they exist in *B* as ions or as molecules). The rate of penetration of K^+ and Na^+ is evident from Table I: H^+ or OH^- also penetrates since HCl passes out quite freely (see page 673) and this is true in much greater degree of citric and formic acids; furthermore *C* becomes more alkaline as the experiment proceeds (e.g. rising from a pH value of less than 6 to 7.5 in Exp. 74, Table I). It is also evident that HCO_3^- or CO_2 penetrates freely: in Model I approximately 80 cc. of CO_2 per hour in the form of CO_2 or HCO_3^- passed from

C into A (i.e., a little less than 2 cc. per square centimeter of surface per hour). It may be that HCO_3^- as such cannot penetrate rapidly²⁹ but it can unite with H^+ and pass out as H_2CO_3 or CO_2 . For con-

TABLE II

Approximate Ratios of Internal and External Activities in the Steady State

	$\frac{H_i^+}{H_o^+}$	$\frac{K_i^{++}}{K_o^+}$	$\frac{Na_i^{++}}{Na_o^+}$	$\frac{\text{HCO}_3^{\dagger}}{\text{HCO}_3^-}$	$\frac{\text{Cl}_o^-}{\text{Cl}_i^-}$	$\frac{G_o^{-\dagger}}{G_i^-}$
<i>Valonia</i>	158.5	41.6	0.18	102.4	0.97	—
Exp. 74, Table I	39.8	9.7	—	0.043	—	39.8
Exp. 66, Table I	79.4	11.2	7.6	0.012	—	79.4

* The activity coefficients for K^+ and Na^+ are from Harned, H. S., *J. Am. Chem. Soc.*, 1922, **44**, 252; 1929, **51**, 416, assuming them to be equivalent to those of Cl in each case.

† Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925-26, **9**, 255. The activity coefficients of HCO_3^- are from Güntelberg, E., and Schiödt, E., *Z. phys. Chem.*, 1928, **135**, 393.

‡ The concentration of G^- (guaiacol ion) was calculated from the hydrolysis equation $G^- = \frac{1.17(10^{-10})}{10^{-14}} (\text{HG}) (\text{OH}^-)$. The value of HG (undissociated

guaiacol) was estimated by observing how much guaiacol was taken up when, for example, in Experiment 74, 0.63 M KHCO_3 was shaken up with an excess of guaiacol. This gave 0.113 M whence $G_i^- = 0.00042$. Taking $G_o^- = KG$, we have $G_o = 0.04$ and $G_o^- \div G_i^- = 95$. If the activity of HG be the same in A and in C then $G_o \div G_i = \text{OH}_o \div \text{OH}_i = 10^{-4.9} \div 10^{-6.5} = 39.8$. The use of concentrations in place of activities and uncertainties in both may account for the difference.

venience in discussion we shall define an ion of this sort as a *penetrating ion*. We may say that all the ions in our experiments are penetrating ions (and this may be the case in *Valonia* since there is very little

²⁹ Since the dielectric constant of guaiacol, which makes up 70 per cent of the non-aqueous mixture, is approximately 12 and that of *p*-cresol 5.6 (cf. International critical tables, New York, McGraw-Hill Book Co., Inc., 1929, **6**, 92) it would seem that there must be some dissociation of the CO_2 in the non-aqueous mixture and hence some passage of HCO_3^- as such.

organic matter in the sap³⁰ and the chief reactant, CO_2 , passes out freely).³¹

The question now arises whether such ions are to be regarded as diffusible in the Donnan sense. The answer to this question depends on definitions: let us consider the following.

(a) If we say that from the Donnan standpoint an ion is diffusible if its concentration diminish in *A* and increase in *C* (or *vice versa*) diffusibility is to be judged solely by changes in *A* and *C*. Hence in the steady state (when there is no change in the composition of either *A* or *C*) all the ions (including those which are rapidly accumulating) might, on this basis, be regarded as indiffusible, although all of them are penetrating ions.

(b) If, however, we prefer to say that with a system not in equilibrium only those ions are diffusible in the Donnan sense whose concentrations in *A* and *C* change in such a way as to approach a Donnan equilibrium we must regard the ions in our experiment as indiffusible since the system does not move toward a Donnan equilibrium even when the CO_2 stops bubbling.

This may be illustrated by an experiment with Model III in which, after the steady state had been reached, the bubbling of CO_2 was stopped and the progress of the system towards equilibrium was observed. When the CO_2 stopped bubbling, *C* contained $0.98 \text{ M KHCO}_3 + 0.35 \text{ M NaHCO}_3$. The system was left to itself except that the stirring was continued (the outside flow was stopped). After 16 days *C* contained $0.20 \text{ M potassium} + 0.22 \text{ M sodium}$ (these were partly organic salts and partly bicarbonate) and the bicarbonate had fallen from 1.33 M to 0.28 M .

During the same period the concentration in *A* increased from 0.05 M to 0.06 M in the case of potassium, from 0.05 to 0.056 M in the case of sodium, and from 0.021 to 0.047 M in the case of bicarbonate. A control experiment showed that practically no CO_2 was absorbed from the air by the solution in *A*, hence the increase in *A* shows that bicarbonate moved from *C* through *B* into *A* (we know from the partition coefficient that the amount of bicarbonate moving into *A* was more than *B* contained at the start). It is therefore evident that a continuation of this process would equalize the concentration of bicarbonate in *A* and *C* and hence

³⁰ The sap consists of KCl and NaCl with a trace of CaCl_2 and MgCl_2 : the organic matter is about 1.4 parts per thousand.

³¹ For entrance of CO_2 into *Valonia* see Brooks, M. M., *Pub. Health Rep.*, U. S. P. H. S., 1923, 38, 1470; Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925-26, 9, 255; Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, 13, 695.

that the system does not move toward a Donnan equilibrium in which bicarbonate cannot escape from *C*.

This is confirmed by another experiment in which 1 liter of fresh guaiacol-*p*-cresol mixture (not shaken with KOH) was placed in *B*. In *C* was placed 100 cc. of 0.63 KHCO_3 and in *A* 2 liters of 0.05 *M* KG. The system (Model I) was left to itself for 28 days (the stirring being continued but with no outside flow). At the end of this period *C* contained 105 cc. the concentrations being 0.16 *M* potassium and 0.063 *M* bicarbonate (hence 0.097 *M* KG). *B* contained 0.023 *M* potassium (mostly KG). *A* contained 0.06 *M* potassium and 0.013 *M* bicarbonate (hence 0.057 *M* KG). All of this bicarbonate (25 millimols) must have moved from *C* through *B* into *A* since practically none was absorbed from the air.

Let us now consider the process of accumulation more in detail. In addition to the inward rate of diffusion of KG, which we may call R_{KG} , we must consider the outward rate of diffusion of KHCO_3 , which we may call R_{KHCO_3} . It is evident that potassium will go on accumulating as long as R_{KG} is greater than R_{KHCO_3} . This might happen even though acid were escaping from *C* into *A* provided the concentration of acid in *C* did not fall so low that R_{KG} ceased to exceed R_{KHCO_3} .³² Evidently equilibrium cannot occur as long as acid is escaping, and it is evident that no equilibrium occurs in our experiments. As far as KG is concerned equilibrium is reached when $(K_i)(G_i)$ is equal to $(K_o)(G_o)$, regardless of the nature of the membrane.

Accumulation while acid is escaping is illustrated by earlier experiments where, after placing KOH in *A* and acid in *C*, the system was left to itself (with no renewal of solutions). We found that potassium entered freely and accumulated (page 673) but at the same time acid passed from *C* into *A*, such acids as carbonic, formic, and citric coming out rapidly (HCl came out more slowly and there was accumulation as described on page 673).

The relation of this to the Donnan principle need not be discussed at length. None of our results conflicts with this principle. If we call the acid inside HZ we may say that the more slowly Z^- diffuses out the more nearly will the situation resemble that treated by Donnan. There is, however, this difference. If Z^- be a non-penetrating ion the system will approach an equilibrium where the composition of *A*

³² A rise in pH value in *C* would hinder the transformation of KG to KHCO_3 thereby lessening R_{KG} .

will not be identical with that of B . But if Z^- and the other ions penetrate even to a slight extent the system will move toward an equilibrium where A and C will be identical in composition (as in our experiments). When the system is prevented from reaching this equilibrium and a steady state is created by constant renewal of Z^- and movement of other substances (including water), there will be differences between A and C in the steady state not predictable by the Donnan equations (it may be remarked incidentally that these equations take no account of the movement of water or of increase in the total number of ions).

Let us now consider other models. MacDougal³³ has described certain kinds. Our model, as previously mentioned, is a modification of that of Irwin.⁴ Models showing accumulation have been described by Netter³⁴ and by Northrop.³⁵ The latter resemble ours more than do those of Netter. For example, in one of Northrop's experiments acetic acid passed through a collodion membrane and reacted with CaCO_3 , giving CO_2 and calcium acetate. This may be compared with our experiment in which KOH passes in and reacts with CO_2 to form water and KHCO_3 . In both cases a reactant was added as a separate phase (solid CaCO_3 , gaseous CO_2) to avoid a large excess of neutralizing material in solution and in both cases the inside contained more reactant than the outside.

Straub³⁶ has recently described models in which an E.M.F. is employed to produce a steady state. In a different sort of experiment oxalic acid was allowed to diffuse out of a porous pot and react with marble (0.1 M LiCl being placed inside and outside) until a steady state was reached. These experiments seem to differ in several respects from ours and we prefer not to attempt any detailed comparison.

9. The model resembles *Valonia* in that very little calcium or magnesium enters the non-aqueous layer as compared with potassium and sodium.

³³ MacDougal, D. T., *Proc. Am. Phil. Soc.*, 1928, 67, 33. MacDougal, D. T. and Moravek, V., *Protoplasma*, 1927, 2, 161.

³⁴ Netter, H., *Arch. ges. Physiol.*, 1928, 220, 107.

³⁵ Northrop, J. H., *J. Gen. Physiol.*, 1929-30, 13, 21.

³⁶ Straub, J., *Chem. Weekbl.*, 1930, 27, 672.

This is shown by the following experiment with Model I. *A* contained 0.1 M KCl, 0.1 M NaCl, 0.1 M CaCl₂, and 0.1 M MgCl₂ (volume 2 liters). *B* contained 1 liter of guaiacol-*p*-cresol mixture; *C* contained 100 cc. distilled water. All three were stirred by glass stirrers. At the end of 154 hours (during which loss of evaporation from *A* and *C* was compensated by adding water) the following concentrations was found in *C*: 0.06 M potassium, 0.03 M sodium, 0.0017 M calcium, and 0.0005 M magnesium.

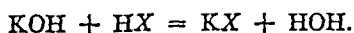
10. The model resembles *Valonia* in that chloride enters more readily than sulfate but the difference is much greater in the case of *Valonia*.

A model of the same dimensions as Model I in which *A* was a mixture of 0.1 M KOH and 0.1 M NaOH and *C* was 100 cc. of a mixture of 0.4 M H₂SO₄ and 0.4 M HCl was allowed to run for 8 days. At the end of this time the chloride and sulfate content of *A* was determined and found to be 0.01 M and 0.0036 M respectively, hence chloride passed from *C* through *B* into *A* about three times as rapidly as sulfate.

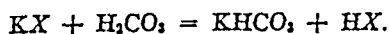
11. The use of a continuous non-aqueous layer to represent the protoplasmic surface seems to be necessary for reasons which have been dealt with elsewhere.³⁷ Since our object is to illustrate the principles governing accumulation in *Valonia* rather than to discover the actual substances concerned, we are at liberty to choose any non-aqueous substance. Of course the fact that certain phenols seem suitable for our purpose does not imply that the protoplasmic surface is composed of these substances.

12. KOH penetrates more rapidly than KCl.³ This is shown by experiments in which 0.05 M KCl is placed in *A* instead of 0.05 M KOH. When this is done the penetration is extremely slow.

Let us now consider certain features of the mechanism of penetration. Since KOH combines at the outer surface of *B* with an organic acid which we may call HX, the reaction may be written



At the inner surface of *B* another reaction takes place,



The molecule of HX thus formed may be presumed to return to the outer surface of *B*, there to react with KOH. The net result is that *A*

³⁷ Osterhout, W. J. V., *Biol. Rev.*, 1931, 6, 402.

loses a potassium ion and C loses a hydrogen ion. This is thermodynamically equivalent to an exchange of K^+ for H^+ , but the mechanism involved may be very different from a simple passage of K^+ and H^+ in opposite directions through the non-aqueous layer. For according to current conceptions potassium must exist in the non-aqueous layer chiefly in undissociated form,³⁸ as molecules or as non-conducting ion pairs which for our present purpose amounts to the same thing. For convenience we shall not try to distinguish between them but we shall speak of such non-conducting ion pairs as molecules.

In order to attribute the major importance to ionic exchange it would be necessary to assume that the mobilities of K^+ and H^+ , for example, are very high as compared with those of molecules of K-guaiacolate and of guaiacol. As the results of Walden³⁹ and others indicate that not over 10 per cent of dissociation is to be expected in substances with so low a dielectric constant,⁴⁰ the velocity of the slower ion of the exchanging pair would have to be at least 100 times as great as that of the corresponding molecule in order to make the total ionic transport in one direction ten times as great as the molecular.

One important feature of the hypothesis is not represented in the model: the fact that in *Valonia* KCl rather than $KHCO_3$ accumulates has been discussed in previous papers where it is suggested that HCO_3^- (passing through the non-aqueous layer as such or as undissociated CO_2 or H_2CO_3) is exchanged for Cl^- from the sea water (which may pass through the non-aqueous layer as such or as undissociated HCl). Our experiments in this connection are not yet complete.

SUMMARY

Inasmuch as attempts to explain accumulation by the Donnan principle have failed in the case of *Valonia*, a hypothesis of the steady state has been formulated to explain what occurs. In order to see

³⁸ This would be true of all salts of potassium, whether organic or inorganic.

³⁹ Walden, P., *Elektrochemie nichtwässriger Lösungen*, Johann Ambrosius Barth, Leipzig, 1924.

⁴⁰ The dielectric constant of *p*-cresol at 24°C. is 5.6 and of supercooled guaiacol is 11.7 (International critical tables, New York, McGraw-Hill Book Co., Inc., 1929, 6, 92).

whether this hypothesis is in harmony with physico-chemical laws attempts have been made to imitate its chief features by means of a model.

The model consists of a non-aqueous layer (representing the protoplasmic surface) placed between an alkaline aqueous phase (representing the external solution) and a more acid aqueous phase (representing the cell sap).

The model reproduces most of the features of the hypothesis. Attention may be called to the following points.

1. The semipermeable surface is a continuous non-aqueous phase.
2. Potassium penetrates by combining with an acid HX in the non-aqueous layer to form KX which in turn reacts with an acid HA in the sap to form KA . Since KX is little dissociated in the non-aqueous layer potassium appears to pass through it chiefly in molecular form.
3. The internal composition depends on permeability, *e.g.*, sodium penetrates less rapidly than potassium and in consequence potassium predominates over sodium in the "artificial sap." The order of penetration in the model is the same as in *Valonia*, *i.e.*, $K > Na > Ca > Mg$, and $Cl > SO_4$, but the quantitative resemblance is not close, *e.g.*, the difference between potassium and sodium, and chloride and sulfate is much less in the model.
4. The formation of KA and NaA in the sap raises its osmotic pressure and water enters.
5. The concentration of potassium and sodium and the osmotic pressure become much greater inside than outside. For example, potassium may become 200 times as concentrated inside as outside.
6. No equilibrium occurs but a steady state is reached in which water and salt enter at the same rate so that the composition of the sap remains constant as its volume increases.
7. Since no equilibrium occurs there is a difference of thermodynamic potential between inside and outside. At the start the thermodynamic potential of KOH is much greater outside than inside. This difference gradually diminishes and in the steady state has about the same value as in *Valonia*. The difference in pH value between the internal and external solutions is also similar in both cases (about 2 pH units).

8. Accumulation does not depend on the presence of molecules or ions inside which are unable to pass out.

One important feature of the hypothesis is not seen in the model: this is the exchange of HCO_3 for Cl^- . Experiments on this point are in progress.

THE OXYGEN CONSUMPTION OF *ESCHERICHIA COLI* DURING THE LAG AND LOGARITHMIC PHASES OF GROWTH

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INTRODUCTION

The oxygen consumption of bacteria has been studied by various methods and by numerous observers. Many of these methods are qualitative and will not be discussed here. The quantitative methods which have been used have been adequately described by Wohlfeil (1930, *a*). In general, most of the quantitative work has been done with thick suspensions of the organisms in saline or Ringer's solution to inhibit growth and reduce the variations due to changes in the number of organisms studied. Inasmuch as rapid multiplication is one of the characteristic processes in the normal life of a bacterium, correlation of respiration and growth presents an interesting problem. Verzar and Bogel (1920), using a Barcroft differential manometer, followed the oxygen consumption and carbon dioxide output of a bouillon culture of *Escherichia coli* over a period of 4 or 5 days but did not record the rate of growth quantitatively.

Novy, Soule and their coworkers (1925) investigated the gaseous metabolism of the tubercle bacillus and other organisms over long periods of time, but growth was followed only qualitatively. Wohlfeil (1930, *b*) determining oxygen consumption by gas analysis studied the respiration of a growing culture of *E. coli* and followed the rate of growth by quantitative methods. His results will be discussed later.

Methods

Oxygen Consumption.—A microrespirometer similar to Fenn's (1927) modification of Thunberg's apparatus is admirably suited to accurate determinations of the oxygen utilization of bacterial cultures, because of its sensitivity over a wide

range of rate changes. The apparatus used in these experiments was a further modification of Fenn's respirometer by Jares.¹ It consists essentially of two flasks, *E* and *C*, (Fig. 1) connected by means of ground glass stoppers and three-way stop-cocks, *S*, to side arms, *A*, opening to the air, and to each end of an even-bored capillary tube, *T*. The respirometer differs from the one used by Jares only in the shape and size of the flasks. The control flask, *C*, may be of any shape but the experimental flask, *E*, is shaped like a short squat Erlenmeyer flask and in addition contains a "well," *W*, made of a short piece of glass tubing fused into the bottom of the vessel which serves as a container for the sodium hydroxide. The shape of the latter flask allows 5 cc. of medium to have a surface area of 25 sq. cm.

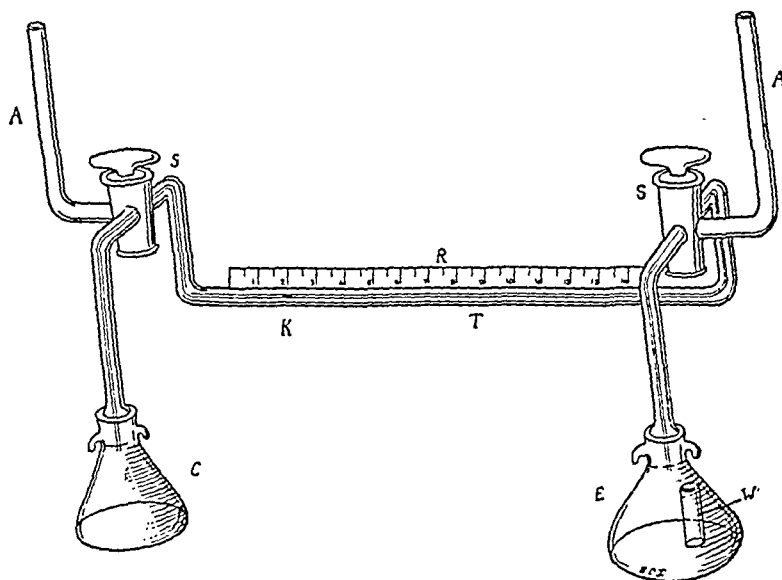


FIG. 1

Calibration of the flasks was done by filling to the level of the stop-cocks with mercury and weighing. Similarly, the capillary was calibrated by weighing the mercury which filled a measured length of the tube. The constants for the apparatus used in the following experiments were:

Vol. of experimental flask =	36.06 cc.
" " control " =	24.11 "
" " capillary tube 1 cm. in length =	8.214×10^{-3} cc.

Only the flasks were autoclaved, as it was found that contamination from the stoppers occurred only rarely, and the purity of the culture was checked hourly by plating methods. A small drop of kerosene, *K*, was placed in the capillary tube and a celluloid rule, *R*, marked in millimeters, was wired to the tube.

¹ To be published.

After placing 1 cc. of sodium hydroxide in the center well, *W*, of the experimental flask, 5 cc. of sterile medium was placed in this flask and 1 cc. in the control flask. The entire apparatus was then immersed to the level of the top of the stop-cocks in a rapidly stirred water bath kept at a temperature of $37.5^{\circ} \pm 0.01^{\circ}\text{C}$. and was shaken at a constant rate by a motor-driven shaking device. The carbon dioxide formed during the experiment was taken up by the sodium hydroxide and as oxygen was consumed by the culture the drop moved toward the experimental flask. Readings of one end of the drop were taken at various intervals by sighting along a plumb-line held near the end of the drop in order to avoid parallax. Determination of the amount of oxygen consumed by the culture was calculated from observation of the distance through which the drop had moved during a definite time interval *t*, according to Fenn's (1928) formula:

$$x = d \left(\frac{V_c + V_e}{V_c + d} \right) \left(\frac{p - y}{p} \right),$$

where *x* = oxygen consumption during time *t*

d = volume of capillary tube through which drop has moved during time *t*

V_c = volume of control flask minus volume of medium

V_e = volume of experimental flask minus volume of medium and sodium hydroxide

p = barometric pressure

y = vapor pressure in both flasks

Since *d* is so small in comparison with *V_c*, for practical purposes it has been dropped from the expression $\frac{V_c + V_e}{V_c + d}$ but of course must be retained elsewhere in the equation.

One of the disadvantages of this method lies in the fact that in order to withdraw samples of the culture for analysis, the apparatus must be removed from the water bath and the flask opened to the air, which disturbs the pressure equilibrium and increases the risk of contamination. Control experiments have shown, however, that if the sample is taken quickly and the apparatus returned to the water bath immediately, 10 minutes is sufficient time for equilibrium to be reestablished.

Medium.—A 2 per cent Difco Bacto-peptone solution in distilled water, containing 0.5 per cent NaCl, pH 7.6, was used in all experiments. As may be expected, the sterile medium *per se* used oxygen at a very slow rate. The rate of oxygen consumption is practically constant over a period of a few hours, but gradually diminishes over a period of 24 hours or longer. The greatest rate observed with this medium was 1.0×10^{-8} cc. of oxygen per minute per cc. of medium. In the early stages of the experiment, when there are comparatively few bacteria present, 50 to 60 per cent of the total oxygen consumption can be accounted for by the medium itself, but within an hour after inoculation this cor-

reaction becomes almost negligible. For this reason, the medium was put into the flask 24 hours before inoculation and the apparatus placed in the water bath and shaken. Readings were taken at various intervals and the rate of oxygen consumption of the sterile medium during the hour preceding inoculation was assumed to be constant throughout the experiment, all calculations being corrected according to this figure. Even though this value may not be correct 4 to 5 hours after inoculation it may be assumed to be constant for the first hour or two, which is the only period in which the value is large enough to cause error.

Organisms.—*Esch. coli* (Strain S) was used in all experiments. The culture was carried along on agar slants or in peptone water until 12 to 20 hours before the beginning of the experiment, when one loopful was inoculated into a large flask of medium identical with the medium in the respirometer. This flask was kept in the incubator until 1 hour before inoculation, when it was placed in the water bath with the respirometer, so that the temperatures of the inoculum and substrate were almost identical. 0.05 cc. of this culture was the usual amount transferred to the experimental flask.

Bacterial Counts.—0.05 cc. of culture was withdrawn at 30 minute intervals throughout each experiment. Plates were streaked for the detection of contamination, and after suitable dilution of the organisms in saline, the count was made in an ordinary Helber hemocytometer. Many of the organisms observed were not separated but in chains, sometimes as many as 8 or 10 occurring in a single chain. By careful focussing, the lines of division were easily seen and the count was made assuming each unit of the chain to be a bacterium. This method of course did not indicate whether the organisms were viable or not, but over the short period of observation—4 hours, all organisms were assumed to have some degree of viability.

Measurements of Size.—A loopful of the culture was smeared on a slide, allowed to dry in air, fixed by heat, and stained with methylene blue. As pointed out by Henrici (1923) the methylene blue method is inaccurate because of distortions of form due to shrinking of the cells. The negative staining method, however, is unsuitable in media containing peptone because of precipitation of the peptone by Congo red. At first, the length and width of each bacterium was measured by a filar micrometer, but it was later found that camera lucida drawings, using an $\times 25$ ocular could be easily and quickly made, and measurements were made from the tracings of the cell outlines. Approximately 100 cells of each smear were measured, the lengths and widths averaged and the area of the average cell calculated assuming the cell to be cylindrical in shape.

In order to prevent selection of the cells to be measured, the smears were mixed and relabeled by another person. Only after the measurements were completed and averaged, were the smears placed in the proper sequence.

Calculations.—All calculations were reduced to a basis of 1 cc. of culture. A slight correction in the formula had to be made after each withdrawal of a sample, since the total volume of the culture was reduced, and the value for V , increased a very slight amount with every reduction in the volume of medium.

It is obvious from the above that the measurement of the rate of oxygen utilization of the culture was not continuous, because of the time allowed for equilibrium to be regained after withdrawal of the sample. For this reason the oxygen consumption per minute per bacterium had to be calculated indirectly, since the actual number of bacteria per cc. of medium was not known during the observed period.

Buchanan's (1930) formula for the determination of the amount of chemical change per single cell per unit time was used as the basis for the calculations. This formula is:

$$m = \frac{2.303 S \log b/B}{t(b - B)}$$

where m = amount of substance produced (or oxygen consumed) per cell per unit time

S = total amount of substance produced (or oxygen consumed) per time t

B = number of bacteria present at beginning

b = number of bacteria present at time t

The formula is based on the assumptions that m is constant throughout the period of observation and that growth is logarithmic. As will be demonstrated later, m is not constant but varies with the phases of the life cycle. For the purpose of calculation m is considered constant for only the relatively short period of actual observation of the movement of the drop, and is calculated separately from the data obtained during each observation period.

Since there is no actual count of the organisms at the time of the first reading of the position of the drop, the value B must be calculated. The formula used for this was as follows:

Let a = number of organisms present at time 0

B = number of organisms present at time x (time of first reading of position of drop)

b = number of organisms present at time $x + t$ (time of second reading of position of drop)

g = generation time during interval $x + t$

$$b = a 2^{x+t/g}$$

$$B = a 2^{x/g}$$

Assuming g to be constant during the relatively short interval $x + t$, and solving for B —

$$\log B = \frac{x}{x+t} (\log b - \log a) + \log a$$

Placing the value for B in Buchanan's equation, m may be calculated. The calculations of the first period in Table I is presented as an example:

The experimental flask contained 5.0 cc. of sterile medium and 0.5 cc. of 1 N NaOH in the well. The control flask contained 1.0 cc. of sterile medium. After

23 hours in the water bath under the same conditions as were present during the experiment, it was found that the drop moved a distance of 0.18 cm. in 60 minutes, a rate of 0.0006 cm. per minute per cc. of medium.

The barometric pressure was 760.4. Calculating according to the formula for the respirometer:

$$0.0006 \times 8.214 \times 10^{-3} \times \left(\frac{23.11 + 30.56}{23.11} \right) \left(\frac{760.4 - 47.9}{760.4} \right) \left(\frac{273}{273 + 37.5} \right) =$$

0.0941×10^{-4} cc. of oxygen consumed per minute per cc. of sterile medium.

(Inasmuch as the CO_2 formed in the control flask was not removed by NaOH and the R.Q. was unknown, this value should more properly be called the medium correction rather than the actual oxygen consumption of the medium).

The experimental flask was then inoculated with 0.05 cc. of a 12 hour culture containing 421×10^6 organisms per cc., giving an initial concentration of 4.17×10^6 organisms per cc. in the 5.05 cc. of medium in the flask. 12 minutes after inoculation the position of one end of the drop was 5.51 on the scale, and 30 minutes after inoculation the reading was 5.70, a movement of the drop through a distance of 0.19 cm. in 18 minutes.

Calculating by the same formula (V , now becoming 30.51 because of the increase in the volume of the medium to 5.05 cc.) the total oxygen consumption was 2.982×10^{-3} cc.

5.05 cc. of sterile medium would account for 0.855×10^{-3} cc. in 18 minutes leaving 2.127×10^{-3} cc. as the oxygen consumption of the organisms *per se*. Reducing this to oxygen consumption per cc. of culture it becomes 0.4212×10^{-3} cc.

At time of inoculation there were 4.17×10^6 organisms per cc. (a). 30 minutes later there were 5.58×10^6 organisms per cc. (b). Calculating the organisms per cc. 12 minutes after inoculation (x):

$$\log B = \frac{12}{12 + 18} (\log 5.58 \times 10^6 - \log 4.17 \times 10^6) + \log 4.17 \times 10^6$$

$$\log B = 6.6708$$

$$B = 4.686 \times 10^6$$

Substituting into Buchanan's formula

$$m = \frac{2.303 \times 0.4212 \times 10^{-3} \log \frac{5.58 \times 10^6}{4.686 \times 10^6}}{18 (5.58 \times 10^6 - 4.686 \times 10^6)}$$

$$= 4.57 \times 10^{-12}$$

= the average oxygen consumption per minute per organism during the period 12 to 30 minutes from inoculation.

All subsequent periods are calculated in the same way.

Eighteen experiments were done and the measurements of the average surface area of the organisms were carried out in eight. Because of the individual variations in each experiment, the results are better considered separately rather than averaged. The tables and graphs of three representative experiments are presented.

TABLE I

Inoculum 0.05 Cc. of a 12.0 Hour Peptone Water Culture Containing 421×10^6 Organisms Per Cc.

(a)

Time from inoculation	No of organisms per cc. $\times 10^4$	Log No of organisms per cc	Average length of organisms	Average diameter of organisms	Average area
min.			cm. $\times 10^{-4}$	cm. $\times 10^{-4}$	sq. cm. $\times 10^{-4}$
0	4 17	6 620	1.83	0.61	4 09
30	5 58	6.747	2 17	0.66	5.19
60	9 05	6.957	2.75	0.80	7.92
90	24 5	7.389	3.22	0.76	8.59
120	67 0	7.826	2.35	0.73	6.23
150	151 9	8 182	2.18	0 64	5.02
180	343.0	8.535	1.97	0.64	4.61
210	900.0	8 954	1.72	0.56	3.52
240	1850.0	9.267	1.42	0.53	2.81

(b)

Time from inoculation	O ₂ consumption per cc. of medium during observed period	O ₂ consumption per min. per cc. of medium	Log O ₂ consumption per min. per cc. of medium	O ₂ consumption per min. per organism
min.	cc. $\times 10^{-4}$	cc. $\times 10^{-4}$		cc. $\times 10^{-12}$
12- 30	0.4212	0.234	5.369	4.57
40- 60	0.9760	0.488	5.688	6.31
70- 90	3.372	1.686	4.227	9.43
100-120	7.296	3 648	4.562	7.47
130-150	14.84	7.42	4.870	6.44
160-180	27.00	13.50	3.130	5.10
190-202	30.72	25.60	3.408	4.44
204-210	17.06	28.43	3.454	3.47
220-230	32.54	32 54	3.512	2.52
232-240	26.24	32.80	3.516	1.95
250-260	40.50	40 5	3.607	

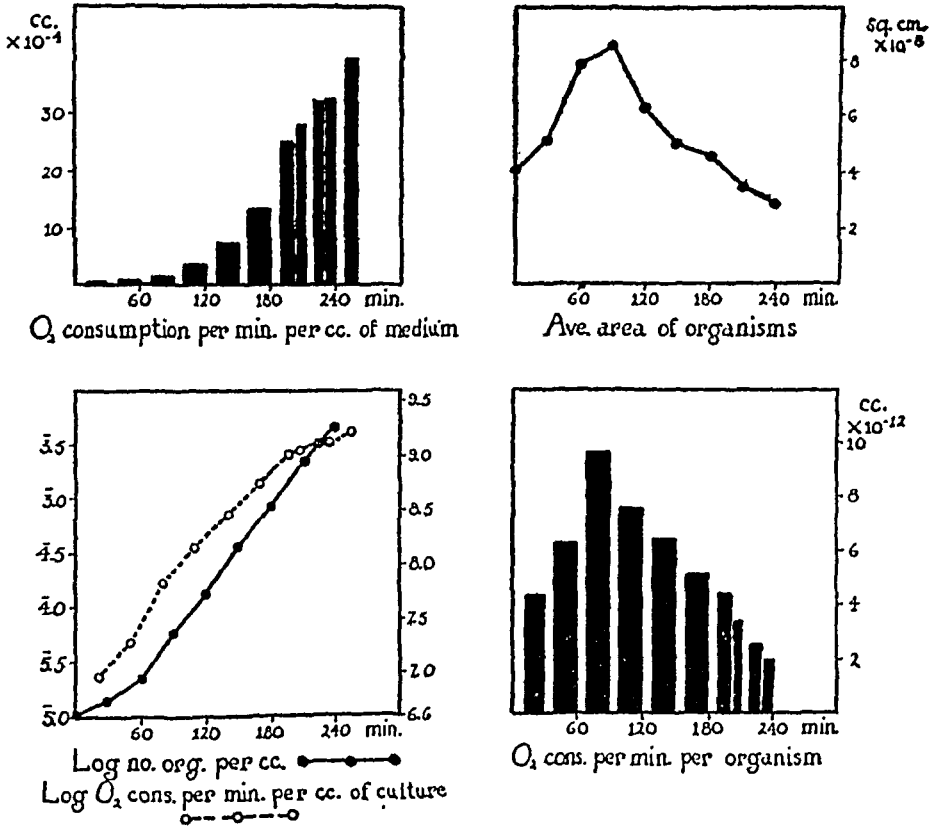


FIG. 2. Graphs of data in Table I.

TABLE II

Inoculum 0.05 Cc. of an 18.0 Hour Peptone Water Culture Containing 503×10^8 Organisms Per Cc.

(a)

Time from inoculation	No. of organisms per cc. $\times 10^4$	Log. No. of organisms per cc.	Average length of organisms	Average diameter of organisms	Average area
min.			cm. $\times 10^{-4}$	cm. $\times 10^{-4}$	sq. cm. $\times 10^{-8}$
0	4.98	6.697	1.95	0.47	3.23
30	9.44	6.975	2.17	0.49	3.71
60	13.85	7.141	3.15	0.65	7.10
90	53.12	7.725	2.67	0.59	5.49
120	125.6	8.099	2.39	0.59	4.98
150	285.0	8.455	2.13	0.58	4.41
180	650.0	8.813	1.87	0.55	3.71
210	1125.0	9.051			
240	2125.0	9.327			
270	3100.0	9.491			

(b)

Time from inoculation	O ₂ consumption per cc. of medium during observed period	O ₂ consumption per min. per cc. of medium	Log O ₂ consumption per min. per cc. of medium	O ₂ consumption per min. per organism
min.	cc. $\times 10^{-4}$	cc. $\times 10^{-4}$		cc. $\times 10^{-12}$
10-30	0.654	0.327	$\bar{5}.515$	4.26
40-60	2.258	1.129	$\bar{4}.053$	9.24
70-90	5.510	2.755	$\bar{4}.440$	7.86
100-120	9.670	4.835	$\bar{4}.684$	5.06
130-150	20.08	10.04	$\bar{3}.002$	4.56
160-180	43.34	21.67	$\bar{3}.336$	4.33
190-210	48.70	24.35	$\bar{3}.386$	2.58
220-240	54.82	27.41	$\bar{3}.438$	1.58
250-270	51.70	25.85	$\bar{3}.412$	0.94
280-300	46.92	23.46	$\bar{3}.370$	

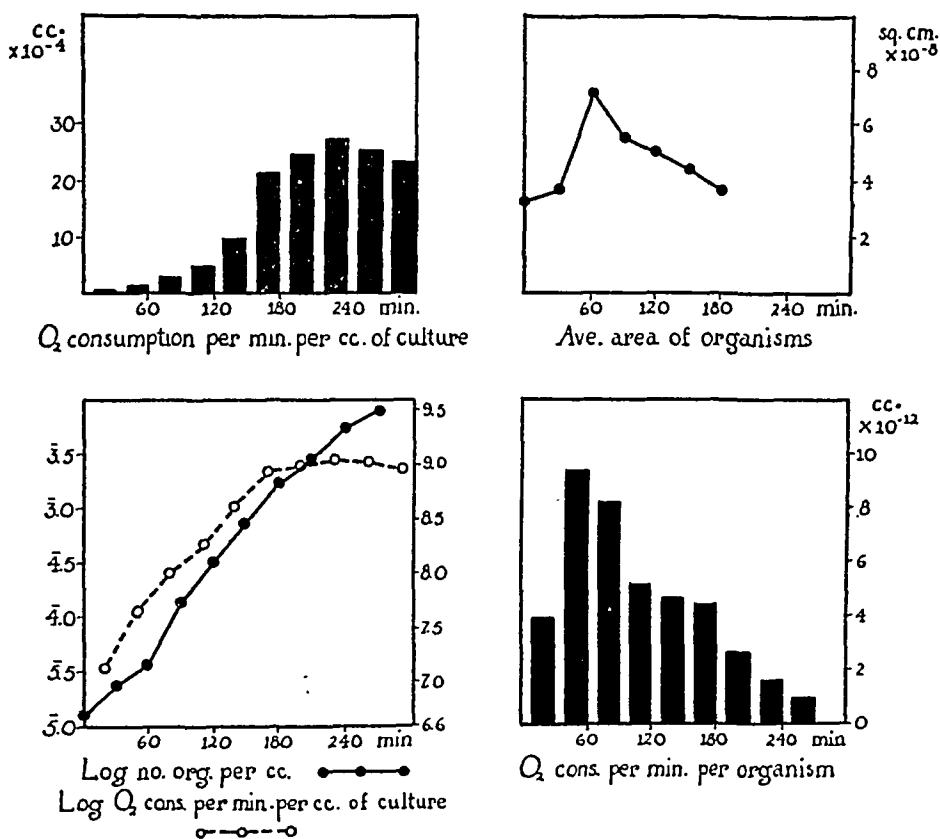


FIG. 3. Graphs of data in Table II.

TABLE III

oculum 0.02 Cc. of a 20.0 Hour Peptone Water Culture Containing 565×10^8 Organisms Per Cc.

(a)

Time from inoculation	No of organisms per cc. $\times 10^4$	Log No. of organisms per cc.	Average length of organisms $\text{cm.} \times 10^{-4}$	Average diameter of organisms $\text{cm.} \times 10^{-4}$	Average area $\text{sq. cm.} \times 10^{-8}$
<i>min.</i>					
0	2.25	6.352			
30	2.50	6.398			
60	3.80	6.580	2.05	0.50	3.61
90	5.5	6.740	2.51	0.52	4.52
120	11.0	7.041	3.45	0.58	6.82
150	28.0	7.447	2.66	0.51	4.67
180	68.75	7.837	2.41	0.53	4.45
210	155.0	8.190	1.80	0.52	3.36

(b)

Time from inoculation	O ₂ consumption per cc. of medium during observed period $\text{cc.} \times 10^{-3}$	O ₂ consumption per min. per cc. of medium $\text{cc.} \times 10^{-4}$	Log O ₂ consumption per min. per cc. of medium	O ₂ consumption per min. per organism $\text{cc.} \times 10^{-11}$
<i>min.</i>				
10-30	0.260	0.130	5.114	5.37
40-60	0.368	0.184	5.265	5.53
70-90	0.600	0.300	5.477	6.17
100-120	1.892	0.946	5.976	10.74
130-150	3.848	1.924	4.284	9.23
160-180	7.790	3.895	4.591	7.54
190-210	14.00	7.000	4.845	5.85

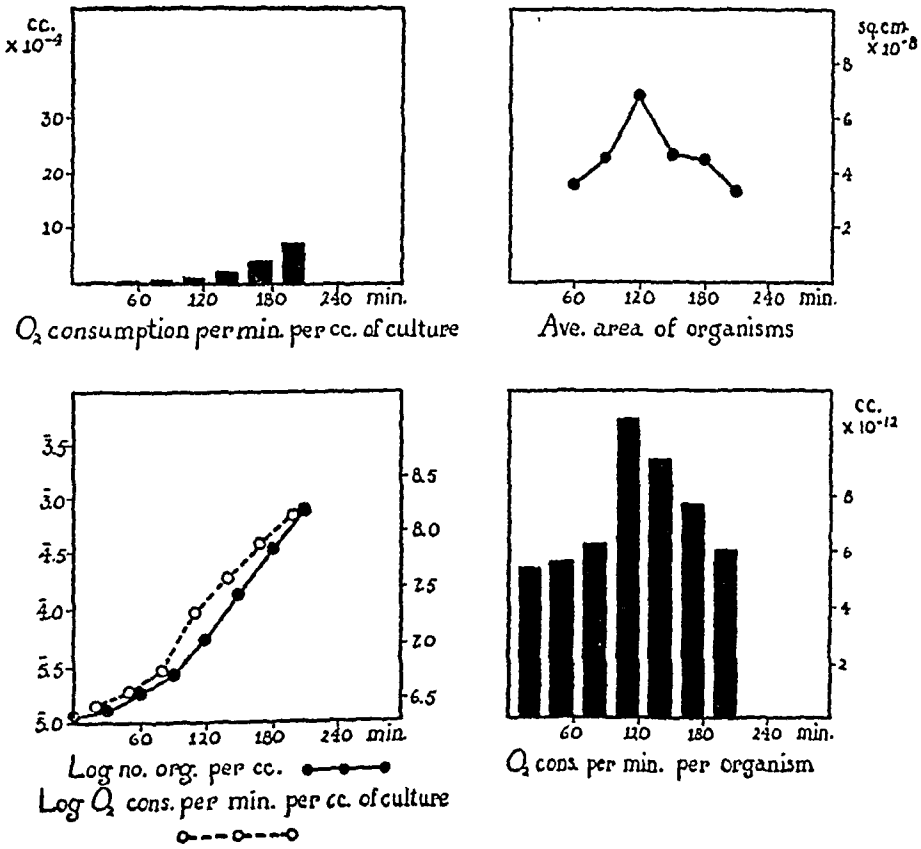


FIG. 4. Graphs of data in Table III.

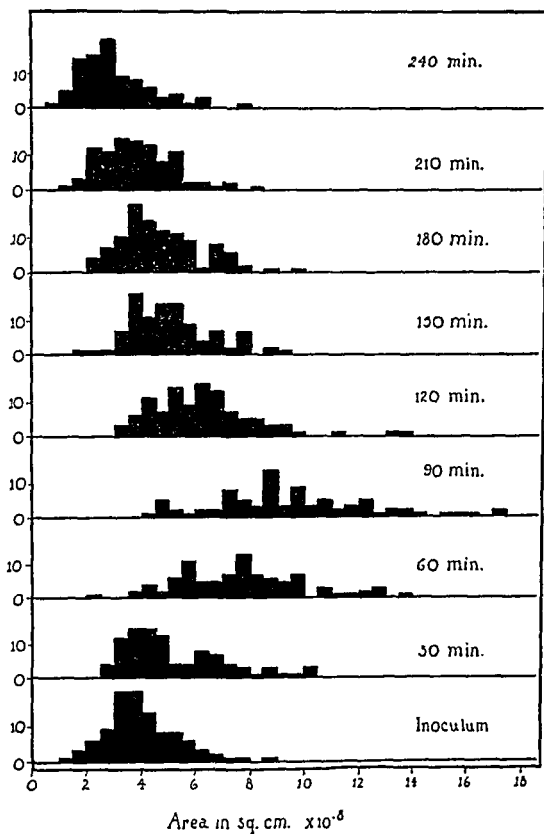


FIG. 5

DISCUSSION

Rate of Growth.—Under the conditions of these experiments, the rate of growth was usually very rapid. In one experiment (Table I, Fig. 2) an average generation time of 23.4 minutes was maintained, the culture 4 hours after inoculation attaining a concentration of 1850×10^6 organisms per cc. This may be contrasted with the cell count of the inoculum which was 421×10^6 organisms per cc. after 12 hours' growth in the same medium but under considerably less oxygen tension in the incubator. Whether this stimulating effect was due to the increased availability of oxygen or to the prompt removal of formed CO_2 by the NaOH was not studied. There were marked differences in the growth rates, which generally varied, as might be expected, with the age and amount of inoculum. The lag phase in these experiments lasted from 1 to $1\frac{1}{2}$ hours before the logarithmic phase began.

Oxygen Consumption.—In general, the rate of oxygen consumption of the culture was proportional to the rate of growth of the cells in the culture, highest values being obtained in those experiments in which growth was most rapid. In nearly all experiments, the rate of oxygen consumption continued to rise throughout the observed period, but in several instances (Table II, Fig. 3), there was a slight falling off in the rate of oxygen utilization near the end of the experiment.

A comparison of the logarithmic curves of the rates of growth and rates of oxygen consumption is interesting. It will be noted that the logarithmic curve of the rate of oxygen consumption becomes a straight line consistently earlier than does the logarithmic growth curve, and in about one-half the experiments (Figs. 2 and 3) this logarithmic phase is apparently present at the time of the first observation—20 minutes after inoculation. The difference between these curves becomes more apparent when the rate of oxygen consumption per organism is calculated.

A period of increased metabolic rate per organism occurred near the end of the lag phase from 1 to 2 hours after inoculation. In Table I, the maximum rate was found 80–100 minutes after inoculation, in Table II it occurred from 40–60 minutes after, and in Table III 100–120 minutes from the start of the experiment. Most of the experiments showed this phase to be during the 80–100 minute period of

observation. The protocol in Table III was from the only experiment in which the maximum was reached as late as 2 hours after inoculation. When the lag phase was prolonged the appearance time of this period of maximum rate of oxygen consumption was delayed.

Bayne-Jones and Rhees (1929) in their study of the heat production of a growing culture of *Esch. coli* noted an increased heat production per cell 2 hours after inoculation. Their figures were based on the total heat production per cell, calculating the total heat produced from the inoculation to time *T*. Recalculating their figures on the basis of the rate of heat production per cell modifies the shape of their curves to only a slight extent, still showing a period of markedly increased metabolic rate per cell in the early stages of growth.

Although the surface-volume ratio of the culture medium in Bayne-Jones' calorimeter was much less than that in the respirometer used in these experiments, and the rate of growth was correspondingly less, it is interesting to compare the absolute values obtained by direct calorimetry with those obtained by the indirect method.

Table 2, page 131, of the paper by Bayne-Jones and Rhees (1929) presents the data obtained on the heat production of the same strain (S) of *Esch. coli* in 2 per cent peptone water. Calculating the heat production from the 1st to 2nd hour (the period of maximum rate of heat production) it is seen that the total heat produced by the 100 cc. of culture was 7.76 gm. cals. The bacterial count at the beginning of this period was 20.5×10^6 organisms per cc. and at the end 45.4×10^6 per cc. Calculating from Buchanan's formula, the value 4.13×10^{-11} gm. cals. per minute per organism is obtained. Since the R.Q. is not known, the average R.Q. of protein oxidation (0.82) may be assumed for the purpose of calculation, which gives 4.825 as the caloric equivalent of 1 cc. of oxygen. Reducing gm. cals. to cc. oxygen, this figure becomes 8.56×10^{-12} cc. oxygen consumed per cell per minute. This is within 15 per cent of the average value obtained by indirect calorimetry during the period of maximum oxygen consumption per cell. Although the exact extent of the oxidative processes as compared with anaerobic processes taking place in the culture under these conditions has not been investigated, the close agreement between the figures obtained by these totally different methods at least suggests

that the cells derive a considerable portion of their energy from an oxidative metabolic process.

Additional evidence toward this point is brought by Walker and Winslow (1932), working on the carbon dioxide and nitrogen output of an aerated culture of bacteria of the colon group. In their abstract they state that "late in the preliminary lag period and in the phase of logarithmic increase the culture produced 40 to 100×10^{-11} mgm. of CO_2 per cell per hour." Reducing these figures to CO_2 per cell per minute they become 3.39×10^{-12} to 8.48×10^{-12} per minute per organism.

The similarity in magnitude of the figures obtained from direct calorimetry, carbon dioxide production, and oxygen consumption by entirely different methods and by different observers is striking.

Wohlfeil (1930, *b*) found that the oxygen consumption per cell decreased with time. When he plotted the relative oxygen consumption per cell as a function of the bacterial count he obtained the same shape of curve as he had previously found in measuring the oxygen consumption of saline suspensions of non-multiplying bacteria in varying concentrations. He concluded from this, that:

"The bacteria respire during growth at the beginning more intensively, not because they need more oxygen for the building up of their body substance, but only because there are fewer microbes present in the unit space and because there is more oxygen available per organism."

There are several possible explanations of his failure to find a low rate of oxygen consumption per cell in the very first stages of growth as compared with the increased rate near the end of the lag phase. One source of error may have been the relatively long periods of time over which the oxygen consumption was measured; *i.e.*, the period of low initial rate per cell may have been so short that it was missed entirely. It is also possible that the failure to correct for the oxygen consumption of the medium *per se*, which has been shown can be as great as that of the bacteria during the initial stages, might have given this apparent high initial rate per cell.

Surface Area.—The variations in size of the organisms in growing cultures have been studied by Henrici (1928), and Jensen (1928). The same phenomenon was noted in these experiments, but with the

added observation that the period of maximum rate of oxygen utilization per cell coincided in every instance with the period of maximum surface area of the organisms. This is brought out in Figs. 2-4, where the maximum surface area appeared at three different time intervals, namely, 90, 60, and 120 minutes after inoculation. Calculations based on the relations of surface area and oxygen consumption have been disappointing as the variations were too great to permit drawing quantitative conclusions. Some of the difficulties faced in determining an average size for the organisms in a single smear will be apparent from a glance at a frequency curve (Fig. 5) obtained from the same experiment as that in Table I. There is a very large "spread" in the size of the cells in a single smear. This is, of course, to be expected when it is considered that a large cell just before division has almost twice the surface area one of the daughter cells will have a few moments later. This great variation prohibits drawing quantitative conclusions on the basis of surface area unless many times as many cells are measured. It is interesting to note that the average area of the cells 4 hours after inoculation was less than that of the 12 hour old inoculum. This may be related to the fact that there were more than four times as many cells per cc. at this time than there were in the inoculum.

The similarity in the shape of the area-time curve and the shape of the curve of the rate of oxygen consumption per organism suggests that the metabolism of the cell is related in some way to the surface area of the cell, but the data presented do not bear this out quantitatively. There are so many other factors of great importance in determining the rate of energy metabolism that further conclusions seem unjustified.

SUMMARY

The oxygen consumption of rapidly growing cultures of *Esch. coli* (S) have been measured by means of Fenn's respirometer.

The rate of oxygen consumption of a growing culture uniformly attains a phase of logarithmic increase before the growth curve of the organisms becomes logarithmic.

The rate of oxygen consumption per cell increases rapidly from the time of inoculation to a point of maximum respiration near the end of the lag phase of the growth curve, followed by the respiratory rate.

The surface area of the average cell when plotted against time passes through a point of maximum surface area which coincides with the point of maximum oxygen consumption per cell.

Figures obtained by different methods, CO₂ output and heat production when reduced to the same units, agree remarkably well.

CONCLUSIONS

The measurement of oxygen consumption of a growing bacterial culture is easily and accurately measured by use of Fenn's respirometer.

Esch. coli (S) in a growing culture passes through a stage of increased metabolism per cell, this stage occurring near the end of the lag period at a time when the individual cells have the greatest size and surface area.

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ON THE INTENSITY-TIME RELATIONS FOR STIMULATION BY ELECTRIC CURRENTS. I*

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Justification is felt in advancing a new analysis of the time-intensity relations of tissue stimulation by electric currents for these reasons: first, the underlying hypotheses are as simple or simpler mechanically than those of previous formulae; second, the agreement with existing experimental data is as good or better; and third, the derivation of the formulae is very easy for all the common types of electrical stimuli without making any approximations.

The derivation is as follows: let the state of excitation or local excitatory process in the tissue under the influence of the stimulus be represented numerically by p and let the rate of attainment of this state be directly proportional to the exciting current or voltage. Further let there be a tendency of the tissue to remain normal leading to a reaction directly proportional to p so that finally,

$$\frac{dp}{dt} = KV - kp \quad (1)$$

In order that the tissue respond to the stimulus it is supposed that p must attain a liminal value h , so that there obtains for adequate direct current stimuli the relation,

$$\int_0^h \frac{kdp}{KV - kp} = -k \int_0^t dt$$

Integration gives finally,

$$\log \frac{KV}{KV - kh} = kt \quad (2)$$

where t is the time during which the stimulus is required to act.

* Partial preliminary report in *Proc. Soc. Exp. Biol. and Med.*, 1932, 29, 615.

If the above hypotheses are correct, equation (2) should give the time-intensity relations for direct current stimulation.

An easy method of testing the equation is derived from noting that as t becomes very great

$$KV - kh \rightarrow 0;$$

but V is now the rheobase, R , whence comes the close approximation

$$KR = kh$$

giving

$$\log \frac{V}{V - R} = kt \quad (3)$$

If experimental values of $\frac{V}{V-R}$ are plotted on semilogarithmic scale against the appropriate times, linearity tests the validity of equation (3).

In Fig. 1 are plotted three sets of data with $\frac{V}{V-R}$ on logarithmic scale against time on natural scale. These data are from Lapicque's book (1926). The three curves, a , b , and c respectively are of data from *Spirogyra*, sciatic gastrocnemius of the frog, and a nerve-muscle preparation of *Helix*. It will be noted that there is fair linearity except for the long times. In these cases, since V is nearly equal to R , a very small change in either makes a large change in $V/(V-R)$, therefore positions of points corresponding to these times cannot be required to conform closely on account of probable experimental errors. For example in c where the value of $V/(V-R)$ diverges most from linearity at long times its value for the last point on the graph is $61/(61-60) = 61$. If the rheobase were in error by ± 1 the resulting values of $V/(V-R)$ would be 30.5 or ∞ respectively. Small changes in V or in the rheobase do not, however, have much effect upon $V/(V-R)$ when V is large compared to R . Linearity can thus be required at short times if equation (3) is fulfilled but its existence at long times depends on extreme accuracy of measurement. It is evident that the curves do not pass through the origin, so an arbitrary constant will

have to be added to equation (3) giving now as the possible relation,

$$\log \frac{V}{V-R} = kt + C \quad (4)$$

where C is the constant whose meaning is considered later.

Equation (4) may be tested numerically, as follows: consider the rheobase R to be a perfect measurement leaving only k and C to be

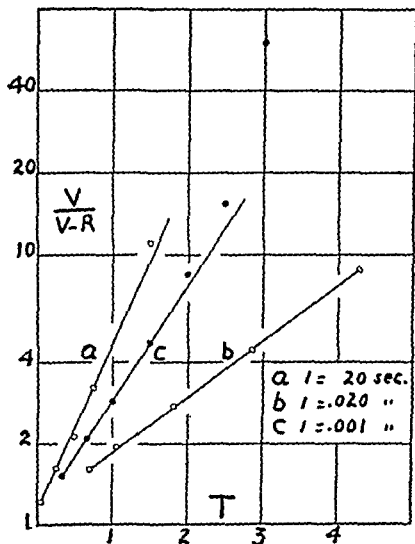


FIG. 1. A convenient scheme for plotting direct current data. *a*, *Spirogyra*; *b*, *Rana*; *c*, *Helix*.

determined. Two sets of V 's and t 's are required for this. It is obvious, of course, that either current data or voltage data may be used equally well without conversion. The sets to be used can best be chosen from a graph like Fig. 1. The data of the first and fourth

TABLE I

(a) <i>Spirogyra</i> , pp. 214-215. $k = 0.03036$; $C = 0.0524$							
t sec.	1	5	10	15	30	100	
V obs.	*5.8	2.6	1.9	*1.45	1.1	1.0	
V cal.	5.8	2.66	1.8	1.45	1.1	1.0	
(b) <i>Helix</i> , nerve-muscle, pp. 214-215. $k = 10.24$; $C = 0.0636$							
t sec.	0.014	0.021	0.036	0.057	0.086	∞	
V obs.	*8.2	6.4	5.0	*4.0	3.5	3.1	
V cal.	8.2	6.5	4.9	4.0	3.5	3.1	
(c) Sciatic gastrocnemius, pp. 214-215. $k = 422.9$; $C = 0.0422$							
t sec.	0.00033	0.00067	0.001	0.0015	0.002	0.0025	0.003
V obs.	*175	115	92	*76	68	64	61
V cal.	175	114.1	91.2	76	68.9	65.1	63.1
(d) Sciatic gastrocnemius, p. 92. $k = 0.07183$; $C = -0.0163$							
t sec.	3	4	6	8	12	16	∞
V obs.	*82	63	49	41	*35	32	30
V cal.	82	64.7	49.6	41.5	35	32.4	30
(e) Sciatic gastrocnemius, p. 96. $k = 497.0$; $C = 0.0664$							
t sec.	0.00033	0.00066	0.001	0.0015	0.002	0.0025	0.003
V obs.	*270	*187	155	126	115	112.5	112
V cal.	270	187	153.4	131.8	122.1	117.3	114.4
(f) Frog stomach. $k = 0.2299$; $C = 0.0842$							
t sec.	0.05	0.10	0.50	1.0	2.0	3.0	10.0
V obs.	41	*30	18	11.8	*9.1	8.3	6.5
V cal.	33.5	30	17.7	12.6	9.1	7.8	6.5
(g) Frog stomach, p. 86. $k = 0.8843$; $C = 0.0763$							
t sec.	0.05	0.10	0.50	5.0	∞		
V obs.	*9.5	7.0	*3.3	2.3	2.3		
V cal.	9.5	7.3	3.3	2.3	2.3		
(h) <i>Vorticella</i> , p. 99. $k = 88.12$; $C = 0.0461$							
t sec.	0.00215	0.00285	0.0036	0.0071	0.0107	0.021	0.050
V obs.	*25	21.5	18.5	14.5	*11.7	10.5	10.5
V cal.	25	21.2	18.5	13.3	11.7	10.6	10.5

TABLE I—*Concluded*

(i) <i>Aplysia</i> , p. 82. $k = 4.663$; $C = 0.1783$									
t sec.	0.006	0.009	0.018	0.027	0.048	0.078	0.157	0.223	∞
V obs.	7.6	*6.2	5.0	4.3	*3.6	3.1	2.7	2.3	2.0
V cal.	6.7	6.2	5.3	4.5	3.6	2.9	2.3	2.1	2.0

* The numbers marked with asterisks are the values of i or V in each case which were used for determining k and C .

† / arbitrary units.

points of Curve a are suitable, for example, as these points both lie on the line going most nearly through all the points. Having determined k and C , and considering the times given to be perfect measurements, it is now easy to calculate what the stimulating voltage should be if equation (4) is a true relation.

This has been done for all the data in Lapique's book in which a rheobase was given, and with much of his recent data (1931, a , b). In Table I are shown the values of i or V both experimental and calculated by means of the equation for the data in Lapique's book. The numbers marked with asterisks are the values of i or V in each case which were used for determining k and C . The calculated values of these have to agree, of course, with the experimental ones.

It will be seen that in most cases the agreement of calculated with experimental values is quite good within the whole ranges. In Case c where the general agreement is poorest a better fit could be obtained by assuming an error in the rheobase, *i.e.*, by making the rheobase 110 say, but since there is no justification for doing this it was thought better to use the value given.

In Case f the calculated value of V is much too low for the shortest time. This does not occur in g which deals with the same kind of tissue, so there is a possibility that the experimental value is too high in f due to some variation of conditions or procedure.

On the whole it is felt that taking into consideration the diversity of types of tissues represented, the general good agreement between the calculated and experimental values points fairly definitely to the conclusion that equation (4) gives the time-intensity relation for the stimulation of tissue by direct currents of constant value as closely as can be expected considering the probable errors of measurement.

Relation to Other Direct Current Formulae

Lapicque (1907) applied the conception of the leaky condenser to the problem of tissue stimulation. The leaky condenser is a mechanism whose action conforms to the differential equation (1), as will now be shown.

If a potential V is applied to a condenser of capacity c in series with a resistance R and shunted by a resistance r the equating of electromotive forces gives,

$$V = Ri + \frac{q}{c}$$

where i is the current through R and q is the charge on the condenser. But,

$$i = i_1 + \frac{dq}{dt} = \frac{q}{cr} + \frac{dq}{dt}$$

where i_1 is the current through the shunt. Thus,

$$\frac{dq}{dt} = \frac{V}{R} - q \frac{(r + R)}{Rcr}$$

which is of the same form as,

$$\frac{dp}{dt} = KV - kp$$

If the excitation process is like the action of a leaky condenser, an experimental confirmation is possible, in at least one way, through the necessary relations of K and k . For then will,

$$K = \frac{1}{R} \text{ and } k = \frac{r + R}{Rcr}$$

which quantities are not independent. The results of Adrian (1) indicate the independence of K and k , *i.e.*, they are against the condenser hypothesis.

The fundamental hypothesis of Hoorweg (1892) may be written,

$$\frac{dp}{dt} = \alpha ic^{-\beta t}$$

where α and β are constants and i is the current, *i.e.*, he supposed the rate of building up of the local excitatory process to be proportional to the current but that the effective current decreased exponentially. A solution for i constant is,

$$\int_0^\eta dp = \alpha i \int_0^t e^{-\beta t} dt, \text{ or, } \log \frac{\alpha i}{\alpha i - \eta \beta} = \beta t$$

where η has the same meaning as the h used here. This solution is the same as equation (3) despite the difference of the hypotheses. It is also equivalent, of course, to Lapicque's equation (1907).

Equation (3) may be written,

$$V/(V - R) = 1 + kt + \frac{k^2 t^2}{2} + \dots \text{ etc.}$$

Neglecting the squared and succeeding terms and multiplying by $V - R$,

$$V = V - R + Vkt - Rkt,$$

or,

$$V = \frac{R}{kt} + R$$

which is of the form of Weiss's law. Weiss's law is related to equation (3) as a first approximation.

Equation (4) may be put in the form,

$$V = (V - R) e^{kt+C} = \frac{R e^{kt+C}}{e^{kt+C} - 1} = \frac{R}{1 - e^{-(kt+C)}}$$

Putting $\theta = e^{-k}$ $\mu = e^{-C}$

$$V = \frac{R}{1 - \mu \theta^t}$$

which is the same as Hill's equation (1910) in its simplified form.

It is generally admitted that Weiss's law, as well as Hoorweg's and Lapicque's equations are inadequate. In regard to Hill's analysis and the present one, since the approximation he used to apply to direct current data is equivalent to the equation derived here, the

direct current data cannot be used to choose between them. The data which fit the one will fit the other. Hill's formulae for other types of stimulation have received very little application. The fact that the present formulae are generally applicable may be due more to their mathematical simplicity than to greater inherent accuracy of representation of the phenomenon. No attempt at a decision in this matter is intended here.

The Constant C

A discussion of the meaning of or the existence of C does not involve the validity of the hypotheses represented by equation (1) for the differential equation of the family of curves.

$$\log \frac{KV}{KV - kp} = kt + C$$

is

$$\frac{dp}{dt} = KV - kp$$

quite independently of the value of C . It is evidently necessary, therefore, to discuss C only in regard to the boundary conditions.

Equation (4) may be written,

$$\log \frac{KV}{KV - kp} = k(t \pm t_0) \quad (4, a)$$

which expressions may be obtained from the integrals of equation (1),

$$\left[\log KV - kp \right]_0^h = -k \int_0^{t \pm t_0} dt$$

The first of these conditions giving C a positive value may be interpreted as a continuation of the process for a time t_0 after the current is off. The second giving C a negative value denotes a delay t_0 , after the stimulus is applied, before the process starts. That the first conditions should be fulfilled is extremely unlikely. The second would represent approximately the case of a delayed rise of the stimulus due to induction. Equation (4, a) would also represent a case in which the calibration of the circuit breaker was in constant error $\pm t_0$. None

of these considerations appear to be useful with the present data, particularly as C may be interpreted in quite a different way.

Equation (4) may also be written,

$$\log \frac{C' V}{V - R} = kt \quad (4, b)$$

where $\log C' = C$. This expression may be obtained by considering the integrals,

$$\left[\log KV - kp \right]_0^{h = \alpha V} = -kt$$

where α is a constant.

This gives

$$\log \frac{KV}{KV - k(h \pm \alpha V)} = kt$$

But when t is large

$$(K \mp k\alpha)V = kh = (K \mp k\alpha)R,$$

where R is again the rheobase voltage. Substituting above for kh ,

$$\log \frac{KV}{(K \mp k\alpha)V - (K \mp k\alpha)R} = kt$$

or,

$$\log \frac{C' V}{V - R} = kt$$

where $C' = K / (K \mp k\alpha)$.

These probably represent the proper boundary conditions. These considerations, which are based on the assumption that the threshold value of the local excitatory process may be influenced by the magnitude of the stimulating current, are perhaps the same that should be applied to the problem of the difference in stimulating powers of "ascending" and "descending" currents. The results of this point of view may be considered.

Since C' of equation (4, b) equals $K/(K \mp k\alpha)$, C which equals \log

C' and is transposed will be positive when $C' > 1$ and negative when $C' < 1$. C is positive when $C' = K/(K + k\alpha)$, but this occurs when the limit of integration is $p = h - \alpha V$, i.e., with a low threshold. But, since low thresholds occur with "descending" currents, C should be positive for "descending" currents and negative for "ascending" currents, providing, however, that h is the same for currents in both directions. If h is different no generalization can be made at present.

It will be seen that the recent data of Lapicque, now to be considered, show that C may disappear with certain electrodes, indicating that the electrodes as well as the direction of the current are a factor. But when C is present it usually changes sign with reversal of direction of the current, as may be expected from present considerations. No conclusions can be drawn in this matter, however, without the investigation of a large number of cases. This presents another problem in the experimental investigation of the effect of electrodes recently reopened by Rushton (1931). Consideration of the data of Table II will illustrate this point.

The data of Table II which consist of a large part of Lapicque's recent work (1931, *a*, *b*) have been handled in the same way as the older data in Table I. Some of his new data whose plots do not give smooth time-intensity curves obviously are combinations of the so-called α and γ types of response. The selection considered here was made, perhaps not always successfully, with a view to avoiding these mixed curves. A point to be noted, which affects the agreement of the calculated and observed voltages at the longer times, is that the rheobases, as Lapicque states, were difficult to determine. A rheobase either too small or too great produces a systematic divergence of observed and calculated voltage for the long times.

Sets *j* and *l* were obtained with one fluid electrode and one stigmatic on normal muscle. The sets differ in that the current is reversed. C exists, is about the same size in each set, but changes sign with change of the direction of the current.

Sets *m* and *n* are on the same muscle as *j* and *l* but with the fluid electrode at the opposite end. C is greater, changes sign with change in direction of the current, but is now positive when the fluid electrode is positive whereas in *l* it was negative when the fluid electrode was positive. It thus appears possible that the sign of C depends on the

TABLE II

(j) (1931, a) p. 200. Frog sartorius, fluid electrode, distal -, $k = 3.24$; $C = 0.0038$						
t sec.	0.014	0.025	0.041	0.100	∞	
V obs.	*17.0	10.0	*7.0	2.1	1.9	
V cal.	17.0	10.7	7.0	3.5	1.9	
(l) as j but fluid electrode + $k = 90.1$; $C = -0.0017$						
t sec.	0.0012	0.0023	0.0045	0.009	0.014	0.025
V obs.	*12.5	8	*5	3.8	3.6	3.3
V cal.	12.5	8.2	5.0	3.6	3.2	3.1
(m) as j same muscle pelvic electrode, fluid, -, $k = 8.33$; $C = -0.0468$						
t sec.	0.014	0.025	0.041	0.100	∞	
V obs.	*14	*8	4	2.5	2.5	
V cal.	14.0	8.0	5.1	3.0	2.5	
(n) as m fluid electrode + and pelvic $k = 79.1$; $C = 0.0448$						
t sec.	0.0012	0.0023	0.009	0.014	0.025	0.100
V obs.	*12	7	*4.0	3.5	3.4	3.2
V cal.	12.0	8.1	4.0	3.5	3.3	3.3
(o) (1931, a) p. 206. Fluid electrode, pelvic, +, $k = 292.3$; $C = 0$						
t sec.	0.001	0.002	0.003	0.005	0.010	0.020
V obs.	*4.5	3.2	2.45	2.3	2.2	2.2
V cal.	4.5	3.0	2.5	2.3	2.2	2.2
(p) As o but position of stigmatic electrode different $k = 359.1$; $C = -0.4752$						
t sec.	0.002	0.003	0.005	0.010	0.100	
V obs.	*7.0	*4.0	3.2	3.2	3.0	
V cal.	7.0	4.0	3.2	3.0	3.0	
(q) As o, stigmatic electrode in new position $k = 289.8$; $C = -0.0939$						
t sec.	0.001	0.002	0.003	0.005	0.050	
V obs.	*16	*9.0	6.5	5.8	5.8	
V cal.	16.0	9.0	6.9	6.1	5.8	
(r) As q but stigmatic electrode + $k = 12.74$; $C = 0.0187$						
t sec.	0.003	0.005	0.010	0.020	0.050	0.100
V obs.	*18.5	13.0	*8.0	4.5	2.6	2.3
V cal.	18.5	13.3	8.0	4.9	2.9	2.3

TABLE II—Continued

(s) (1931, a) p. 209 two fluids electrodes $k = 53.8$; $C = 0.0$							
t sec.....	0.0023	0.0045	0.009	0.014	0.025	∞	
V obs.....	*20	13.0	7.0	5.9	5.1	5.0	
V cal.....	20	11.7	7.4	6.1	5.2	5.0	
(t) As s current reversed $k = 46.6$; $C = 0.0$							
t sec.....	0.0023	0.0045	0.009	0.014	0.025	∞	
V obs.....	*19	11	7.2	5.8	5.0	4.2	
V cal.....	19.0	11.0	6.8	5.4	4.5	4.2	
(u) As s but current at 30° to muscle $k = 14.48$; $C = 0.0$							
t sec.....	0.0045	0.009	0.014	0.025	0.100	∞	
V obs.....	11.0	*5.8	3.9	2.9	1.5	1.5	
V cal.....	10.8	5.8	4.0	2.7	1.6	1.5	
(v 1) Sciatic fluid electrode (1931, b) p. 237. $k = 227.5$; $C = 0.0677$							
t sec.....	0.0006	0.0015	0.0025	0.0045	0.010	0.040	0.100
V obs.....	*2.8	1.7	*1.3	1.2	1.0	1.0	1.0
V cal.....	2.8	1.64	1.3	1.2	1.05	1	1
(v 2) $k = 229.1$; $C = 0.0556$							
t sec.....	0.0006	0.0015	0.0025	0.0045	0.010	0.040	0.100
V obs.....	*3.6	1.9	*1.7	1.4	1.3		
V cal.....	3.6	2.2	1.7	1.4	1.3		
(v 3) Mean k of $v_1 v_2 = k = 228$; $C = 0.1642$							
t sec.....	0.0006	0.0015	0.0045	0.010	0.040	0.100	
V obs.....	*2.7	1.8	1.4	1.4	1.4	1.3	
V cal.....	2.7	1.84	1.39	1.3	1.3	1.3	
(w 1) Descending current (1931, b) p. 237. $k = 587$; $C = -0.0257$							
t sec.....	0.0006	0.0015	0.0025	0.0045	0.010	0.100	
V obs.....	*7	*4.3	3.8	3.7	3.7	3.7	
V cal.....	7.0	4.3	3.8	3.71	3.7	3.7	
(w 2) Ascending current. $k = 392.6$; $C = 0.1893$							
t sec.....	0.0006	0.0015	0.0025	0.0045	0.010	0.100	
V obs.....	*7.2	*5.4	4.7	4.5	4.5	4.5	
V cal.....	7.2	5.4	4.8	4.55	4.5	4.5	

TABLE II—*Concluded*

(w 3) Ascending. $k = 384.7$; $C = 0.0813$						
t sec.....	0.0006	0.0015	0.0025	0.0045	0.010	0.100
V obs.....	*4.2	*2.8	2.5	2.3	2.3	2.2
V cal.....	4.2	2.8	2.4	2.25	2.2	2.2
(w 4) Descending. $k = 377.0$; $C = 0.1612$						
t sec.....	0.0006	0.0015	0.0025	0.0045	0.010	0.100
V obs.....	*2.2	*1.6	1.4	1.3	1.3	1.3
V cal.....	2.2	1.6	1.41	1.3	1.3	1.3

* The numbers marked with asterisks are the values of i or V in each case which were used for determining k and C .

direction of the current through the muscle, not on which type of electrode is cathode. This seems to be contradicted in q , r , however.

Sets o , p , q , and r are on the same muscle, the stigmatic electrode being cathode in the first three but placed, in o , on the outside of the ventral part of the muscle, in p , on the middle of the same side, and in q , on the middle of the opposite (back) side. It will be observed that $C = 0$ for o but is quite large for p , smaller and negative for q . This indicates that the existence of C may depend on electrodes insofar as different types of electrodes will cause the current to flow through the tissue in different directions relative to its structure. In regard to time constants it will be observed that the k of o is equal, approximately, to that of q .

The sets, s , t , and u using fluid for both electrodes have $C = 0$ in all cases. Changing the direction of the line joining the electrodes to 30° from the axis of the muscle, lowered the time constant k but made no change in C .

The sets v_1 , v_2 , and v_3 are on the same sciatic nerve (frog) with fluid electrodes. C is not zero as with fluid electrodes on muscle. The constant k of v_3 was, on account of the incompleteness of the data, taken from the mean of those of v_1 and v_2 . The value so obtained is apparently suitable. w_1 , w_2 , w_3 , and w_4 are on still the same nerve after an interval, the electrodes being the same. It will be seen that the k 's of w_2 , w_3 , and w_4 are approximately the same although the C 's are quite different. The same thing is true of v_1 , v_2 , and v_3 . This indi-

cates that the C 's and k 's are not simply related. It may be pointed out that although the time constants k of v_1 , v_2 , and v_3 are approximately equal, the chronaxie will differ on account of differences of the C 's. The same consideration is applicable to w_2 , w_3 , and w_4 .

No attempt can be made from these data to determine the relations of C and k to the type of electrodes used. The data suffice to show, however, that the magnitudes of both these factors have to be taken into account in choosing a method of stimulation, and it is hoped that the electrode problem will be simplified greatly by the fact that it can be examined in terms of the two quantities C and k . The fact that C can be made zero is extremely important as the results of experiments satisfying this condition are much more easily related mathematically. It is also important in that it indicates that C represents something apart from the essential mechanism, because it is not to be expected that the nature of the response process can be altered by a suitable choice of electrodes.

The significance of the data and considerations up to this point may be summed up as follows: integrals of equation (1), which represents the fundamental hypotheses used here regarding the growth of the local excitatory process, represent quite adequately the time-intensity relations for direct current stimulation. The upper limit of integration, *i.e.*, the liminal value of p necessary for the stimulus to be adequate is not in general constant, but is a function of the voltage. The form of this function imposed by the data is *threshold* $= h \pm \alpha V$. There is some indication that the sign of αV depends on the direction of the current, but the matter is further complicated by the fact that α becomes zero with certain electrodes, showing that its value may depend on electrodes as well. The quantity αV may be a measure of the condition known as electrotonus. It represents at least a similar phenomenon, *i.e.*, a raising or lowering of the threshold by the current flowing through the tissue, in this case by the stimulating current itself.

Stimulation by Breaking Constant Currents

The fact that the threshold is dependent on the voltage suggests that break stimuli may be explained on this basis. There may be a certain threshold, h , say, at which the local excitatory process is

adequate when there is no current flowing through the tissue. When there is a current flowing the threshold is $h \pm \alpha V$. Let $h \pm \alpha V$ be greater than h_0 and let the current have raised p to a value between h_0 and $h \pm \alpha V$. Excitation will now be accomplished when the current is stopped if the threshold drops more quickly than the local excitatory process decays, or rather if the threshold drops quickly enough to overtake p before it decays beyond h_0 . This suggestion has little value until the threshold problem has been further investigated but it has some qualitative experimental basis other than the dependence shown here of the threshold on the current, and it is worthy of consideration in that it offers a possibility of explaining break stimuli without invoking additional phenomena.

The discussion of the last paragraph was concerned with break stimuli in general. The problem of determining the least effective break stimuli for given durations has been considered experimentally by Laugier (1921). His data are not available here except for two cases taken from a paper by de Almeida (1931) nor has his paper been seen. These two sets were selected by de Almeida as being those which best fitted his formula. They may, however, be typical.

In Table III the results of applying equation (4) to these cases are given. The calculated voltages marked with asterisk are the same as the observed from which the constants were calculated.

It will be seen that the agreements, particularly in the first set, are not as good as those in Table I, although they are good up until fairly long times. It appears that the voltage tends then to become too low for the relation. This point is illustrated by using the voltage 2.00 at 0.086 seconds as rheobase for an additional set of calculated values. It will be seen that this makes the agreement better. There is no reason to believe, however, that the measured rheobase is not correctly given. The only conclusion to be drawn assuming equation (4) to be true is that in Case 1 the excitability of the nerve became greater with the longer stimulating times.

The values of k are much smaller than those obtained with excitation at the cathode. The C 's are large and positive. These correspond to integrals of equation (1) of the type,

TABLE III

(1) Sciatic gastrocnemius, excitation at the anode. $k_1 = 50.4$; $k_2 = 53.69$; $C_1 = 0.0765$; $C_2 = 0.0811$												
t_{sec}	0.0005	0.001	0.002	0.0036	0.0050	0.0072	0.0086	0.0122	0.0179	0.0287	0.050	∞
V obs.....	9.95	7.50	5.50	4.20	3.55	3.05	2.75	2.45	2.30	2.15	2.10	1.90
V calc.....	9.10	*7.50	5.67	4.24	3.58	2.99	*2.75	2.38	2.12	1.96	1.90	1.90
V calc.....	9.09	*7.50	5.68	4.27	3.62	3.03	2.80	*2.45	2.20	2.05	2.03	2.00
(2) Sciatic gastrocnemius, excitation at the anode. $k = 40.1$; $C = 0.1066$												
t_{sec}	0.0036	0.0072	0.018	0.0286	∞							
V obs.....	49.0	35.00	25.25	23.75	21.50							
V calc.....	*49.0	35.97	*25.25	22.77	21.50							

* The calculated voltages marked with asterisk are the same as the observed from which the constants were calculated.

i.e., the threshold in each case is a constant quantity less a quantity varying as the voltage. This may not be significant but the smallness of k probably is.

It is unsafe to draw many conclusions from these two cases but they indicate the probability that equation (1) represents the growth of the excitatory process at the anode as well as at the cathode.

The Chronaxie

Using Lapicque's definition of chronaxie one obtains by putting $V = 2R$ in (4),

$$\log \frac{2R}{2R - R} = kt + C,$$

which can be written

$$\log 2 - \log \beta = kt \text{ where } \log \beta = C;$$

thus

$$t = \tau = \text{chronaxie} = \frac{1}{k} \log \frac{2}{\beta} \quad (5)$$

or chronaxie is inversely proportional to k . It is interesting that the definition of chronaxie should make it proportional to the reciprocal of the rate of return to normal of the tissue per unit state of excitation.

In order to make chronaxie the type of measure of excitability intended by those who employ it, it will be seen that C must be the same for all the chronaxies to be compared. Conditions giving $C = 0$ are much to be preferred.

The Summation of Inadequate Stimuli

Let the stimulus be sufficient to raise p to θk only, where $\theta < 1$. From equation (1) the local excitation will not return to zero immediately but will decay according to the equation,

$$\frac{dp}{dt} = -kp$$

or,

$$p = \theta h e^{-kT}$$

i.e., p will decay exponentially, and if a second stimulus is applied soon enough an appreciable part of θh will still remain and addition will occur.

The simplest case to consider is that in which the stimuli are all equal and the intervals between the stimuli equal in duration to the stimuli. Let the stimulating voltages be V and the durations t_1 . For the first stimulus,

$$\log \frac{KV}{KV - kp} = kt_1$$

whence,

$$p = \frac{KV}{k} (1 - e^{-kt_1})$$

But p decays in the interval according to the equation,

$$\frac{dp}{dt} = -kp$$

$$\left[\log p \right]_{\frac{KV}{k} (1 - e^{-kt_1})}^p = -kt_1$$

and p at the end of the first interval is given by,

$$p = \frac{KV}{k} (1 - e^{-kt_1}) e^{-kt_1} = \frac{KV}{k} (e^{-kt_1} - e^{-2kt_1})$$

For the second stimulus,

$$\left[\log (KV - kp) \right]_{\frac{KV}{k} (e^{-kt_1} - e^{-2kt_1})}^p = -kt_1$$

or,

$$KV - kp = \{KV - KV (e^{-kt_1} - e^{-2kt_1})\} e^{-kt_1}$$

or,

$$p = \frac{KV}{k} \{1 - e^{-kt_1} + e^{-2kt_1} - e^{-3kt_1}\}$$

For the decay after the second stimulus,

$$\left[\log p \right] \frac{KV}{k} \{ 1 - e^{-kt_1} + e^{-2kt_1} - e^{-3kt_1} \} \approx -kt_1$$

or,

$$p = \frac{KV}{k} \{ e^{-kt_1} - e^{-2kt_1} + e^{-3kt_1} - e^{-4kt_1} \}$$

For the third stimulus,

$$p = \frac{KV}{k} \{ 1 - e^{-kt_1} + e^{-2kt_1} - e^{-3kt_1} + e^{-4kt_1} - e^{-5kt_1} \}$$

Similarly after n stimuli,

$$kp = KV \{ 1 - e^{-kt_1} + e^{-2kt_1} - \dots - e^{-(2n-1)kt_1} \} \quad (6)$$

The bracket is a geometric series whose common ratio is $-e^{-kt_1}$. The sum of a large number of terms will be given by,

$$\frac{1}{1 + e^{-kt_1}}$$

For one adequate stimulus,

$$kh = KV_1 (1 - e^{-kt_1}), C \text{ being neglected.}$$

For n stimuli to be adequate where n is large,

$$kh = KV_1 \times \frac{1}{1 + e^{-kt_1}}$$

where V_1 and V_2 are the liminal voltages for a single stimulus and multiple stimuli, respectively. Equating these expressions,

$$\frac{V_1}{V_2} = \frac{1}{1 + e^{-kt_1}} \times \frac{1}{1 - e^{-kt_1}} = \frac{1}{1 - e^{-2kt_1}} \quad (7)$$

which gives the ratio of the threshold voltages for a single direct current shock to that for many repeated shocks, each of the same duration as the single shock. As far as is known there have been no experiments to test these results. The similar problem arising with

repeated condenser discharge stimuli, however, has been investigated. It will be considered later.

Supernormal Excitability

It was shown by Adrian (1920) that the time-intensity curve for the supernormal phase of excitability as compared to that for resting excitability is merely displaced along the current axis. It is evident from equation (3) that this involves only a change in K as multiplying V and R by a constant does not change the right hand side of the equation. This is of interest here as it shows that one of the postulated processes can be altered without a change of the other, *i.e.*, the direct action of the current is facilitated without altering the rate of the counter action toward normal and without altering the liminal value h of p . As was previously pointed out, these results are against the leaky condenser hypothesis.

SUMMARY

Formulae are derived for the time-intensity relations for stimulation by direct currents using the following hypotheses: first, the current produces an excitatory effect whose rate of growth is proportional to the voltage; and second, the tissue reacts toward the normal state at a rate proportional to the amount of excitation. If p represents the local excitatory process numerically, the hypotheses are represented by the differential equation

$$\frac{dp}{dt} = KV - kp$$

where K and k are constants and V the applied voltage. For the stimulus to be adequate it is assumed that p must be built up to a certain liminal value. It appears as a deduction from the data that this liminal value is a function of the voltage of the form $h \pm \alpha V$ where h and α are constants. α is zero or negligible for certain electrodes. αV is a measure of electrotonus or a similar phenomenon. Experimental data are discussed and are shown to agree satisfactorily with the derived formulae for stimulation both at the anode and cathode.

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ON THE INTENSITY-TIME RELATIONS FOR STIMULATION BY ELECTRIC CURRENTS. II*

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In Part I of the present discussion (Blair, 1932) it was shown that the data for stimulation by direct currents are adequately represented by integrals of the equation,

$$\frac{dp}{dt} = KV - kp \quad (1)$$

where p and V represent the magnitudes of the local excitatory process and the stimulating voltage respectively and K and k are constants. The threshold value of p was found to depend on the voltage so that the upper limit of integration was given by $p = h_0 \pm \alpha V$ where h_0 and α are constants. The sign and magnitude of α depended on the method of stimulation. Under some conditions it was zero in which case the threshold was independent of the voltage. In considering now other forms of electrical stimuli in the light of the same hypotheses it is usually assumed for convenience that the threshold is constant.

Condenser Discharges as Stimuli

The potential q/c of a discharging condenser is given by,

$$\frac{q}{c} = \frac{q_0 e^{-\frac{t}{cr}}}{c} \quad (2)$$

where q_0 and c are respectively the initial charge and capacity of the condenser, r is the resistance of the circuit through which the discharge is taking place, and t the time.

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Substituting in (1) for V from (2) gives,

$$\frac{dp}{dt} = \frac{Kq_0 e^{-\frac{t}{cr}}}{c} - kp \quad (3)$$

which is the differential equation for stimulation by condenser discharges. Its solution is,

$$\begin{aligned} p &= e^{-\int k dt} \frac{Kq_0}{c} \left\{ \int e^{\int k dt} e^{-\frac{t}{cr}} dt + C \right\} \\ &= e^{-kt} \frac{Kq_0 r}{crk - 1} \left\{ e^{\frac{crk-1}{cr} t} + C \right\} \end{aligned}$$

When $t = 0$, $p = 0$, therefore $C = -1$ giving,

$$p = \frac{Kq_0 r}{crk - 1} \left\{ e^{-\frac{t}{cr}} - e^{-kt} \right\} \quad (4)$$

It is further assumed that the tissue responds to the stimulus when p attains a liminal value h ; thus the general equation of stimulation is given by,

$$h = \frac{Kq_0 r}{crk - 1} \left\{ e^{-\frac{t}{cr}} - e^{-kt} \right\} \quad (5)$$

It is necessary to discuss the maximum value of p as a response will be obtained only under conditions such that the maximum value of p just equals or tends to be greater than h . p is a maximum when $dp/dt = 0$, i.e., when,

$$\frac{Kq_0 r}{crk - 1} \left\{ -\frac{e^{-\frac{t}{cr}}}{cr} + ke^{-kt} \right\} = 0$$

or,

$$t = \frac{cr}{1 - crk} \log \frac{1}{crk} \quad (6)$$

On the basis of the hypotheses this time t should be the time at which a response is elicited if the stimulus is just adequate. In other words, equation (6) should give the "*durée utile*," of the discharge.

In Table I are given all the measurements by the Lapicques of the *durée utile* which could be found (1926, 1907, *b*). The page numbers given with the *Helix* data refer to Lapicque's book. The constant "*k*" was calculated in each case from the data marked with asterisks.

TABLE I

Tissue	Durée utile	R.C. $\times 10^4$	<i>k</i>	Time calc.	<i>C</i> ₁
<i>Helix</i> (Lapicque, p. 122)	0.042	*59 0	14.254	0.042	103
	0.026	*39 5	32 85	0 026	142
	0.016	22 5		0 0163	132
	0.012	14.0		0.0122	112
	0 0065	5.6		0.0076	90
<i>Helix</i> (Lapicque, p. 121)	0 038	*50	41.61	0 038	94
	0 013	*25	95 83	0 013	100
	0 009	15		0.0082	106
	0.0043	5		0 0043	92
Sciatic gastrocnemius (<i>Compt. rend. Soc. biol.</i> , 1907, 62, 701)	0.00181	*70	298.4	0.00181	204
	0 00154	*7.0	687 2	0 00154	170
	0.00135	3.5		0.00147	155
	0 00093	1 4		0.00091	142
	0 00063	0.7		0.000602	129

* Values used to determine *k*. Lower value of *k* is to base *c*.

The method of doing this may best be shown by an example: *Helix* (Lapicque, page 122).

$$0.042 = \frac{0.059}{1 - 0.059 k} \log \frac{1}{0.059 k}$$

$$0.026 = \frac{0.0395}{1 - 0.0395 k} \log \frac{1}{0.0395 k}$$

multiplying by the reciprocal of the factor of the log in each case to leave only the log factors on the right hand side and then subtracting,

$$\frac{(1 - 0.059 k) 0.042}{0.059} - \frac{(1 - 0.0395 k) 0.026}{0.0395} = \log \frac{0.0395}{0.059}$$

which can easily be solved for "*k*." A better value of "*k*" would perhaps be the mean value from all the data of each set but this was not determined.

It will readily be seen that the *durées utiles* as calculated from equation (6) agree as well as can be expected with the measured values.

The value of "*p*" at its maximum will be given by substituting the value of *t* from (6) into (3) when $dp/dt = 0$, i.e.,

$$p \text{ max. } h = \frac{Kq_0 e^{-\frac{1}{1-cr\bar{k}} \log \frac{1}{cr\bar{k}}}}{c}$$

which reduces to

$$p \text{ max. } = Krq_0 (cr\bar{k})^{\frac{cr\bar{k}}{1-cr\bar{k}}} \quad (7)$$

The value of *p* max. can also be obtained by substituting *t* from (6) into (4), i.e.,

$$\begin{aligned} p \text{ max. } &= \frac{Kq_0 r}{cr\bar{k} - 1} \left\{ e^{-\frac{1}{1-cr\bar{k}} \log \frac{1}{cr\bar{k}}} - e^{-\frac{cr\bar{k}}{1-cr\bar{k}} \log \frac{1}{cr\bar{k}}} \right\} \\ &= \frac{Kq_0 r}{cr\bar{k} - 1} \left\{ cr\bar{k}^{\frac{1}{1-cr\bar{k}}} - cr\bar{k}^{\frac{cr\bar{k}}{1-cr\bar{k}}} \right\} \end{aligned} \quad (8)$$

It may easily be shown that (7) and (8) are equivalent expressions. If *p* max. = *h* the general equation of stimulation in terms of *q*, *c*, and *r* is given by

$$p \text{ max. } = h = Krq_0 (cr\bar{k})^{\frac{cr\bar{k}}{1-cr\bar{k}}}$$

or for any given tissue the conditions for effective stimulation if *q*, *c*, or *r* vary is given by

$$\text{Constant} = rq_0 (cr\bar{k})^{\frac{cr\bar{k}}{1-cr\bar{k}}} \quad (9)$$

or the equivalent expression derived from (8).

The arithmetic involved in checking these equations is very cumbersome unless "*k*" is determined separately from data giving the *durées utiles* or, as will later be seen, from chronaxie. In Table I the numbers *C*₁ are proportional to the constant of equation (8) for each set of data. The *k* used was derived from the *durées utiles*. Since one is dealing here with high powers of numbers and since small changes

in the exponents make comparatively large changes in the resulting figures, such close agreements are not to be expected here as between the *durées utiles*, calculated and measured. It will be observed, however, that in spite of this the values of C_1 in each set are of the same order of magnitude. The agreement could probably be improved by small adjustments in k , but it may also be necessary to make the threshold a function of the voltage as it was with direct currents in most cases. This does not affect the *durée utile* so it has not been considered. It is to be expected that apart from experimental conditions, the threshold will be less influenced by the voltage of the stimulus with condenser discharges than with direct currents because the voltage is decreasing and is relatively low at the time of the response. It will be seen, however, that if this condition has to be put in equation (9) it can easily be done from (2) and (6).

Chronaxie

Let it be assumed that the rheobase potential is determined by direct current stimuli. From equations (2) and (4) of Part I it follows that the rheobase voltage is given by

$$V = \frac{kh}{K} \quad (10)$$

and twice the rheobase by

$$V = \frac{2kh}{K}$$

From (3) when p is a maximum and equal to h ,

$$kh = \frac{Kq_0c^{-\frac{1}{cr}}}{c} \quad (11)$$

Putting

$$\frac{2kh}{K} = V = \frac{q_0}{c}$$

one obtains

$$t = cr \log 2,$$

but this is by definition, the chronaxie for condenser discharges. Let τ_c be this chronaxie so that,

$$\tau_c = cr \log 2 \quad (12)$$

For direct currents it was found that chronaxie,

$$\tau = \frac{1}{k} \log \frac{2}{\beta} \quad (13)$$

It will be of interest to find the ratio of these two chronaxies.

Since (6) is also true for chronaxie one may put

$$t = \tau_c = cr \log 2 = \frac{cr}{1 - crk} \log \frac{1}{crk}$$

giving,

$$(crk - 1) \log 2 = \log crk \quad (14)$$

which is satisfied when $(crk - 1) = 0$ or $cr = \frac{1}{k}$

i.e.,

$$\tau_c = \frac{1}{k} \log 2.$$

This result however is trivial, as may be ascertained by considering its consequences in any of equations (6), (7), and (8).

$crk = 2$ is also a solution of (14) whence

$$\tau_c = \frac{2}{k} \log 2 \quad (15)$$

Dividing (13) by (15),

$$\frac{\tau}{\tau_c} = \frac{\frac{1}{k} \log \frac{2}{\beta}}{\frac{2}{k} \log 2}$$

or,

$$\tau = \frac{\log \frac{2}{\beta}}{2 \log 2} cr \log 2$$

or,

$$\tau = \frac{\log 2 - C}{2} cr \quad (16)$$

which gives the chronaxie as defined originally for direct current in terms of cr . Lapique by experiment has determined this relation to be, on the average,

$$\tau = 0.37 cr \quad (17)$$

From (16) and (17),

$$\log 2 - C = 0.74$$

$$-C = 0.7400 - 0.6932 = 0.05 \text{ approx.}$$

The values of C determined from Lapique's direct current data, Table I of Part I, range from 0.084 to -0.016 , only one being negative. These have to be multiplied by 2.30, the conversion factor of common to Napierian logarithms, giving 0.18 to -0.037 in order that they may be compared with the value found above. Although the one negative value is close to that calculated from (16) and (17), the positive values would give the τ to cr ratio lower than Lapique's experimental value, the lowest being about $0.26 cr$ while the lowest experimental value was $0.31 cr$ (1926, page 324).

It will be observed that if $C = 0$, equation (16) gives

$$\tau = 0.3466 cr,$$

a value very near to Lapique's mean value 0.37 from experiment.

In recapitulation it may be said, that on the bases of the hypotheses used, it has been found that with the stimulus from a condenser discharge the local excitatory process in tissue rises to a maximum and then decreases if the maximum value is not equal to the liminal value h . If the maximum value is at least equal to or tends to be greater than h a response will ensue. If it is just equal to h the ensuing response occurs at the time the maximum is attained. This time is the predicted *durée utile* of the discharge. The close agreement between the predicted and measured values of the *durées utiles* indicates strongly the validity of the hypotheses.

Fig. 1 is a graphical representation of the discharge of the condenser along with the curve of the growth of the local excitatory process p according to equation (4) for the data of the first line of Table I. The dotted continuation of the p curve represents the value it would have had, had not the maximum been adequate. It will be noticed that the

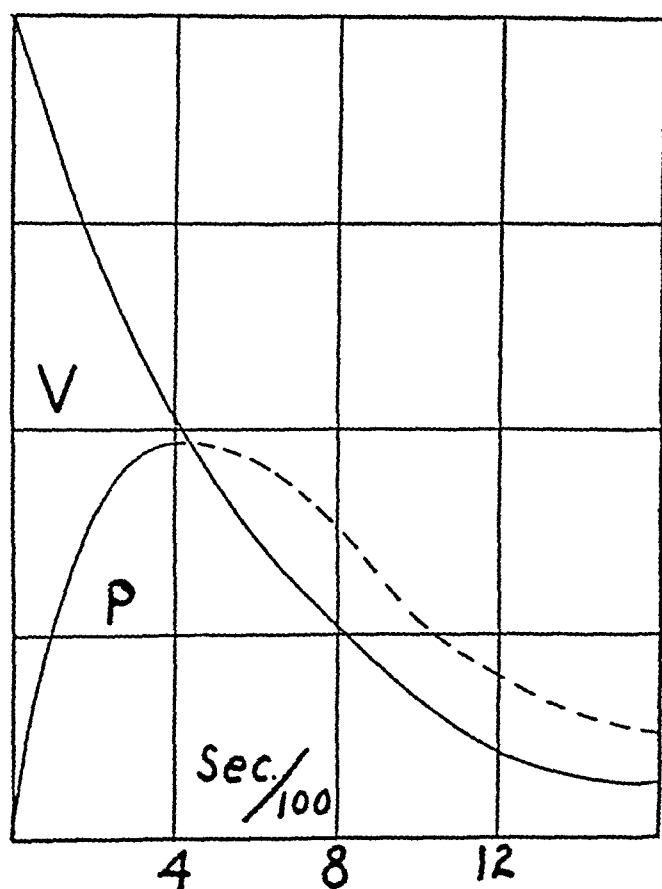


FIG. 1. Graphic representation of the stimulus and response for the first line of Table 1.

top of the p curve is fairly flat. This lack of sharpness indicates that a substantial error may easily be made in the measurement of the *durée utile*. Had the maximum value of p been inadequate it will be observed that even at 0.15 seconds after the beginning of the discharge the comparatively slow decay of p still leaves about one-third of the maximum left. This is of interest as regards the summation of in-

adequate stimuli. The rate of decay of p is, of course, slower in this case than after a direct current stimulus, as here the current is still acting after p commences to decrease.

The Summation of Condenser Discharge Stimuli

For condenser discharge stimuli there was obtained

$$p = e^{-kt} \frac{Kq_0 r}{crk - 1} \left\{ e^{\frac{crk-1}{cr}t} + C \right\}$$

which gives, if $p = 0$ initially, equation (4), i.e.,

$$p = \frac{Kq_0 r}{crk - 1} \left\{ e^{\frac{-t_1}{cr}} - e^{-kt} \right\}.$$

If this p is inadequate at its maximum its value at a time t_1 will be given by

$$p = \frac{Kq_0 r}{crk - 1} \left\{ e^{\frac{-t_1}{cr}} - e^{-kt_1} \right\}$$

Let a similar stimulus be given now at time t_1 which will give

$$p = e^{-kt} \frac{Kq_0 r}{crk - 1} \left\{ e^{\frac{crk-1}{cr}t} + C \right\},$$

where now

$$p = \frac{Kq_0 r}{crk - 1} \left\{ e^{\frac{-t_1}{cr}} - e^{-kt_1} \right\}$$

when $t = 0$;

i.e.,

$$C = e^{\frac{-t_1}{cr}} - e^{-kt_1}$$

or,

$$p = \frac{Kq_0 r}{crk - 1} \left\{ e^{\frac{-t_1}{cr}} + e^{\frac{-t_1}{cr}} - e^{-kt_1 - kt} \right\}$$

If this is again inadequate after time t_1 , again,

$$p = \frac{Kq_0 r}{crk - 1} \left\{ e^{\frac{-t_1}{cr}} + e^{\frac{-t_1}{cr} - kt_1} - e^{-2kt_1} \right\}$$

Similarly for the third stimulus,

$$p = \frac{Kq_0 r}{crk - 1} \left\{ e^{\frac{-t}{cr}} + e^{\frac{-t_1}{cr} - kt_1} + e^{\frac{-t_1}{cr} - kt_1 - kt} - e^{-2kt_1 - kt} \right\}$$

If again this is inadequate p is given after another interval t_1 by

$$p = \frac{Kq_0 r}{crk - 1} \left\{ e^{\frac{-t_1}{cr}} + e^{\frac{-t_1}{cr} - kt_1} + e^{\frac{-t_1}{cr} - 2kt_1} - e^{-3kt_1} \right\}$$

Similarly in a time t_1 after the n 'th stimulus

$$p = \frac{Kq_0 r e^{\frac{-t_1}{cr}}}{crk - 1} \left\{ 1 + e^{-kt_1} + e^{-2kt_1} + e^{-3kt_1} + \dots + e^{-(n-1)kt_1} - \frac{e^{-nkt_1}}{e^{\frac{-t_1}{cr}}} \right\}$$

If n is large the two final terms will be small and may be considered equal giving

$$p = \frac{Kq_0 r e^{\frac{-t_1}{cr}}}{crk - 1} \left\{ 1 + e^{-kt_1} + e^{-2kt_1} + e^{-3kt_1} + \dots + e^{-(n-2)kt_1} \right\}$$

Let $e^{kt_1} = \theta$, then,

$$p = Kq_0 r e^{\frac{-t_1}{cr}} \left\{ 1 + \frac{1}{\theta} + \frac{1}{\theta^2} + \frac{1}{\theta^3} + \dots + \frac{1}{\theta^{n-2}} \right\}$$

Lapicque (Evans, 1930) by considering that the local excitatory process dropped to $\frac{1}{q}$ of its maximum value between stimuli, arrived at the conclusion that for n stimuli,

$$p = M \left\{ 1 + \frac{1}{q} + \frac{1}{q^2} + \dots + \frac{1}{q^{n-1}} \right\}$$

where M is the maximum value of p due to the first stimulus. This expression agreed (Evans, 1930) with the experimental values of the Chauchards. Evidently the expression derived here is equivalent except that the interpretation of $\frac{1}{\theta}$ is not quite the same as Lapicque's $\frac{1}{q}$.

It will be observed that the magnitude of successive terms depends on k as well as the interval t_1 . Since k is large for excitable tissue and

small for relatively inexcitable, very short intervals will have to be used for excitable tissue to get the additive effects obtainable for fairly long intervals on inexcitable tissue.

For any given tissue short intervals, *i.e.*, small values of t_1 , increase both the factor $e^{\frac{-h}{\sigma}}$ as well as the factors in $\frac{1}{\theta}$. It follows that the voltage threshold will be low with rapid repetition of stimuli and will increase as the interval becomes longer approaching finally the threshold for a single stimulus.

Currents of Linear Rise

There are two cases of currents increasing linearly which may be considered: the first being that in which the circuit is broken while it is still rising and the second that in which the current is caused to rise to a certain value and then allowed to flow steadily at that value for a given time.

The differential equation of the first case is evidently given by

$$\frac{dp}{dt} = KV_0 t - kp \quad (19)$$

where V_0 is the gain of voltage per second. The solution is,

$$p = KV_0 e^{-kt} \left\{ \int e^{kt} t dt + C \right\}$$

which finally reduces to,

$$p = \frac{KV_0}{k} \left\{ t - \frac{1}{k} (1 - e^{-kt}) \right\} \quad (20)$$

Again assuming that for the stimulus to be effective p must attain a liminal value h , the general equation for effective stimulation is given by,

$$kh/K = \text{Constant} = V_0 \left\{ t - \frac{1}{k} (1 - e^{-kt}) \right\} \quad (21)$$

As $t \rightarrow \infty$ evidently $V_0 t$, which is the liminal voltage, tends to become equal to kh/K , *i.e.*, to the rheobase for constant direct current, as reference to equation (10) will show. For shorter times the liminal

voltage will be greater than kh/K . The conclusion to be drawn is that any rate of rise no matter how small will eventually stimulate.

In the second case after the current has reached its maximum value it can be treated as a constant direct current. Therefore,

$$\frac{dp}{dt} = KV_o t_1 - kp$$

where t_1 is the time taken in reaching the maximum. Integrating

$$\left[\log (KV_o t_1 - kp) \right]_{\frac{KV_o}{k} \left\{ t_1 - \frac{1}{k} (1 - e^{-kt_1}) \right\}}^p = -kt$$

where the lower limit of p is derived from equation (20). This expression on simplification gives

$$p = \frac{KV_o}{k} \left\{ t_1 - \frac{1}{k} (e^{-kt} - e^{-k(t+t_1)}) \right\}$$

and for effective stimuli,

$$\text{constant} = kh = KV_o \left\{ t_1 - \frac{1}{k} (1 - e^{-kt_1}) e^{-kt} \right\} \quad (28)$$

where t is measured from the top of the rise of the current, *i.e.*, t is the duration of the constant current which is added to the initial varying current.

This equation indicates that if t_1 is small compared to t and t is large there exists the approximation,

$$KV_o t_1 = kh = \text{constant},$$

or since $V_o t_1$ is the liminal voltage one may say that the liminal voltage is nearly constant independently of the rate of rise under these conditions. Since kh/K is the rheobase potential this is equivalent to saying that in measuring the rheobase the mode of initial rise does not change the result providing the time of rise is a small part of the total time. Lopicque (1926), page 133 says in this regard, "But it can be said that up to a certain limit, variable with each excitability, the duration of the period of establishment is practically irrelevant." Lopicque (1926) also mentions the finding of Fick on *Anodonta*, that

the liminal voltage did not have to be raised until the time of rise of the current exceeded 10 seconds.

The extensive data of Keith Lucas (1907) on this subject were obtained with linearly rising currents of the second kind, *i.e.*, they would be represented, if at all, by equation (22). He plotted his results as in Fig. 2, with the liminal voltages as ordinates and the time of rise as abscissae ($V_0 t_1$ in terms of equation (22) against t_1). He found usually, in these graphs, that $V_0 t_1$ remained constant or increased but slightly until the current gradient reached a certain minimum. With gradients less than this minimum no excitation at all could be evoked even

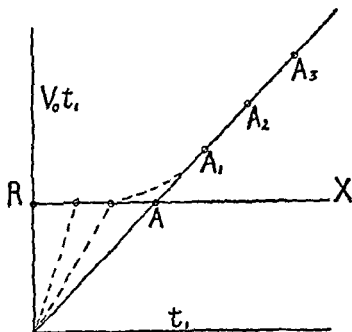


FIG. 2. The results of Keith Lucas with linearly rising currents

though the current was raised greatly above the rheobase. Referring to Fig. 2 the rheobase is represented by RX . Currents which rose to the value R were effective provided they attained the value R within the interval of time RA . If they did not reach the value R within this time they did not excite at all.

It should be remarked that the points A_1 , A_2 , A_3 do not indicate, as they appear to at first sight, that the liminal voltage has to be increased as the time of application of the liminal gradient is increased. Obviously if the currents for A_1 , A_2 , and A_3 had stopped rising at A they would still have been adequate. The only use of these points

is to determine the liminal gradient. What they really indicate is that when the minimal gradient is being used to excite, continuing the rise beyond the rheobase is equivalent to stopping at the rheobase.

Some of Keith Lucas's data indicate a gradual approach to the minimal gradient as represented by the dotted line in Fig. 2. Unfortunately the interval *RA* was not well investigated, not at all with nerve, as his fastest gradient was very near the minimal. With muscle the minimal gradient was slow enough to allow his apparatus to give more than one point in the region *RA*. In this case (1907, page 268) the graphs show the liminal voltage very nearly constant up to the minimal gradient.

It appears that the conclusion to be drawn from Keith Lucas's experiments is that the rheobase is adequate or very nearly adequate no matter how it is linearly attained as long as it is attained within a certain time. If it is not attained within this time raising the voltage still further is of no avail. In other words, the least adequate stimulus is the rheobase whether it is attained abruptly or along a gradient. When the gradient is too low for the rheobase to be effective no voltage is effective. At the liminal gradient the rheobase is just as effective as any higher voltages.

Referring again to equation (22) it will be remembered that its prediction is a constant threshold equal to the rheobase for any rate of rise providing the steady current attained is continued indefinitely. If the steady current is not sufficiently prolonged the liminal voltage will increase somewhat above the rheobase. Evidently equation (22) represents the results of Keith Lucas for all adequate stimuli. It does not explain why gradients less than the minimal are ineffective. A complete theory will have to do this, but as far as is known at present it may be some phenomenon quite apart from the local excitatory process but which is also evoked by the passage of the stimulating current which causes this sudden lack of response.

It will be seen that experiments of the type represented by equation (22) are strictly speaking not investigations of the effect of current gradients, except insofar as they determine the minimal gradients. Evidently this information will have to be obtained by using currents which are cut off while still rising, or from rising and falling currents such as those used by Lapicque (1926). No data on this question ap-

pears to have been obtained using rising currents alone, so equation (21) cannot be tested at present. Like (22) it will not explain a liminal gradient, if such a gradient appears, and it probably will. If equation (21) can be tested experimentally it probably will be necessary usually in doing so to make the threshold depend on the voltage. This is easily done. With equation (22) no different conclusions can be drawn by inserting this condition.

Currents of Exponential Rise

Since an exponentially rising potential can be represented by

$$V = V_0 (1 - e^{-\gamma t}),$$

where V_0 is the terminal potential, the differential equation for p is

$$\frac{dp}{dt} = KV_0 (1 - e^{-\gamma t}) - kp \quad (23)$$

and its solution,

$$pk = KV_0 \left\{ 1 - \frac{ke^{-\gamma t}}{k - \gamma} + \frac{\gamma e^{-\gamma t}}{k - \gamma} \right\} \quad (24)$$

where k is the liminal value of p for response.

The determination of k from these equations alone is also extremely difficult. Data used for easily testing them will have to give means of determining k from direct current or condenser discharge measurements. None of the existing data appear to be suitable.

As with currents of linear rise, equation (24) indicates that when t is very great V_0 becomes equal to the rheobase voltage. This conclusion is probably untrue as it is with linear currents.

It seems that no very definite conclusions can be reached at present in regard to the validity of the equations for currents increasing with time. Fortunately, however, although existing data are unsuitable the required measurements for checking the equations can be obtained by usual procedures.

Alternating Currents

Let the applied potential be of the form $V = V_0 \sin \omega t$ where ω is 2π times the frequency and V_0 the maximum voltage.

The differential equation of the local excitatory process is then given by,

$$\frac{dp}{dt} = KV_o \sin \omega t - kp \quad (25)$$

and,

$$p = e^{-\int k dt} KV_o \left\{ \int e^{\int k dt} \sin \omega t dt + C \right\} \quad (26)$$

This may be simplified by the method of integration by parts, giving finally

$$p = \frac{KV_o}{k^2 + \omega^2} \left\{ k \sin \omega t - \omega \cos \omega t + \omega e^{-kt} \right\} \quad (27)$$

Two sets of maxima of p may be considered, the one including the transient term ωe^{-kt} and the other after the transient term has become negligible. In the first case,

$$\frac{dp}{dt} = 0 = \omega k \cos \omega t + \omega^2 \sin \omega t - k \omega e^{-kt}$$

These maxima may account for the short tetanus observed when a high frequency circuit is applied to tissue even though the threshold for prolonged tetanus has not been reached.

In the second case,

$$\frac{dp}{dt} = 0 = k \omega \cos \omega t + \omega^2 \sin \omega t$$

whence,

$$\tan \omega t = -\frac{k}{\omega}$$

or,

$$t = \frac{1}{\omega} \tan^{-1} -\frac{k}{\omega}$$

Substituting in (27) and ignoring the transient term,

$$p = \frac{KV_o}{k^2 + \omega^2} \left\{ \frac{k^2}{\sqrt{k^2 + \omega^2}} + \frac{\omega^2}{\sqrt{k^2 + \omega^2}} \right\} = \frac{KV_o}{\sqrt{k^2 + \omega^2}}$$

If p at its maximum is adequate, *i.e.*, equal to h ,

$$h = \frac{KV_0}{\sqrt{k^2 + \omega^2}} \quad (28)$$

which is the general equation for tetanizing stimulation by alternating currents. When ω is large compared to k there will exist the approximation $V_0 = \frac{h}{K}\omega$, or the applied voltage at its maximum is proportional to the frequency. This is a conclusion which has also been reached from experiment by Asher (1923) for high frequencies.

As the data on alternating current are not very extensive measurements were made to determine the validity of equation (18).

Apparatus and Method

The source of current used was the low frequency oscillator of the General Radio Company, a vacuum tube oscillator giving very pure sine waves of frequencies from 25 to 70,000. The preparation sciatic gastrocnemius of the frog, was mounted in a moist chamber. The electrodes consisted of two test-tubes each of 1 cm. diameter placed 1 cm. apart and vertically. The tubes were filled with Ringer's solution and the nerve laid across the open end so that the part traversing each tube was completely immersed. Silver chloride electrodes connected the liquid electrodes to the oscillator. The use of non-polarizable electrodes is probably quite unnecessary, but it is unlikely that they will do any harm. A non-inductive resistance of about 2,000,000 ohms in series with the nerve was used in all experiments. The applied voltage was measured on a cathode ray oscillograph. The scale is arbitrary but the numbers given are almost exactly equal to twice the actual voltage. The accuracy of the voltage measurements was in general to about 0.2 or 0.3 of the unit. The method used to determine the threshold was to raise the voltage gradually, stimulating at intervals for short times until the least voltage for a steady contraction was attained. The condition of steady contraction was determined visually without the use of a lever.

In all the experiments but *d* the measurements were made starting at the high frequencies and going to low frequencies. *d* was taken after about forty readings had been made at different frequencies lower than 400. The method of determining constancy of excitability was to make a measurement at frequency 1000 occasionally. If the reading at 1000 changed appreciably the experiment was stopped. This is not an entirely adequate test as shown by the Experiments *e* and *f*. These were done on the same preparation in the order, *e*, *f*. It will be observed that the threshold for high frequencies has become much lower during the interval of about 1 hour separating the two sets. This effect is encountered in nearly every case but the increase of excitability is usually much less and so slow that a single complete

set of readings can be taken without changes greater than the usual variations in successive readings for the same frequency. In *b* for instance the range from 25,000 to 8000 was repeated after going through the complete set. In spite of both twitch and tetanus readings having been taken previously, two of these frequencies gave identical readings the second time and the remainder were sometimes greater and sometimes smaller than the original.

Experimental Results

It will be observed that on putting the constant h/K in equation (28) equal to A and squaring,

$$V_o^2 = A^2 k^2 + A^2 \omega^2 \quad (29)$$

i.e., V_o^2 is a linear function of ω^2 . This provided a convenient means of checking the experimental results. It was surprising to find when this was done that although equation (29) represented the voltage frequency relation extremely well, particular constants A and k applied to limited regions of frequency only, *i.e.*, the graphs of the relation of equation (29) turned out to be straight lines having abrupt changes of slope at various points on the frequency scale.

In Table II are given the data obtained using several preparations for frequencies above 300 or 400. Lower frequencies than this are not considered here as they are in the region in which the voltage-frequency relation may be very variable due to the existence of optimal frequencies (see for example: Renquist and Koch, 1930). The columns containing A and k respectively give the values of these constants for each frequency range. These constants were determined in each case from those observed voltages which are marked with asterisks. Those particular voltages were chosen from the $V_o^2 - \omega^2$ graphs, being values which fell on the straight lines going most nearly through all the points plotted for each range. The column headed *voltage calculated* was obtained by solving equations (29) for V_o using the A 's and k 's previously determined. This method was adopted to show the fit of equation (29) as the range of ω^2 is too large to plot on one scale.

It will be observed that in each range the theory and experiment agree extremely well, which indicates that the threshold voltage throughout the whole range of the frequencies used is that function of the square of the frequency predicted by equation (29). The theory

does not, however, predict changes in the constants. Such a situation was quite unexpected and no reasonable explanation of the effect can be offered. It was expected that if equation (28) applied at all, it

TABLE II

[illegible]

TABLE II—*Concluded*

Exp.....	(d)			(e)			(f)		
Frequency		Voltage			Voltage			Voltage	
		Obs.	Calc.		Obs.	Calc.		Obs.	Calc.
30,000							<i>A</i>	76.0	79.0
25,000							0.0004021	*67.0	67.0
20,000							<i>k</i>	55.0	55.3
18,000									
16,000				<i>A</i>	88.0	89.2	55,630	*46.2	46.2
14,000				0.0007909	*80.5	80.5			
12,000				<i>k</i>	72.0	72.1			
10,000				51,180	64.0	64.1	<i>A</i>	38.0	34.4
9,000					62.3	60.3	0.0003292	*33.2	33.2
8,000					56.3	56.7	<i>k</i>	31.5	32.1
7,000					53.3	53.4	83,480	30.5	31.1
6,000					50.5	50.3		30.0	30.2
5,000					*47.5	47.5		29.3	29.4
4,000					43.0	45.1		*28.7	28.7
3,500					43.0	42.6		28.0	28.4
3,000				<i>A</i>	*40.0	40.0	<i>A</i>	*27.3	27.3
2,500	<i>A</i>	36.5	37.6	0.001284	38.5	37.7	0.0006173	26.0	26.5
2,000	0.002191	*31.5	31.5	<i>k</i>	35.5	35.7	<i>k</i>	26.0	26.0
1,800	<i>k</i>	29.3	29.0	24,800	*35.0	35.0	40,020		
1,600	6,969	27.5	26.8		33.4	34.4			
1,400		24.5	24.6	<i>A</i>	30.2	32.3			
1,200		*22.5	22.5	0.003363	*28.5	28.5			
1,000		20.6	20.6	<i>k</i>	25.5	24.8		*25.0	25.0
900		18.5	19.7	3,863	24.0	23.0	<i>A</i>	24.0	24.8
800		17.8	18.8		22.0	21.3	0.003555	22.5	23.0
700	0.003251	*17.0	17.0		19.5	19.7	<i>k</i>	*21.3	21.3
600	<i>k</i>	15.7	15.3		18.2	18.2		20.5	19.7
500	2,828	13.3	13.8		17.0	16.8	4,068	18.0	18.3
400		*12.3	12.3		*15.5	15.5		*17.0	17.0
300		10.5	10.6						

* Values used to determine *A* and *k*.

would do so fairly well for a limited range of frequencies, but would perhaps diverge gradually at higher frequencies due to the electrical skin effect or to other purely electrical effects arising from the complex

structure of the nerve trunk. The equation, however, appears to represent the data just as well at high as at low frequencies. There appears to be no question that each range is distinct and linear in $V_o^2 - \omega^2$. It is possible, of course, to represent smooth curves approximately by series of straight lines, but if this were being done here systematic differences should appear between the voltages observed and calculated and it is unlikely that the constants A whose squares give the slopes of the line segments would change so greatly in magnitude in going from one segment to the other.

It was pointed out previously that Experiments *e* and *f* were on the same preparation. It will be seen that in these experiments the A 's and k 's both change, although those for the lowest frequency range are about the same in both cases. The procedure of these two experiments was adopted to show that the changes in constants encountered on going through a large frequency range were not due to the properties of the electrical circuit used, as, if they were, the change should always occur at the same frequencies.

The conclusion to be drawn from the values of k observed in these experiments is that the tissue used may become much more excitable, as measured by the time constant, to high frequency currents. The values of k for the highest frequencies are of the order of 100 times those for direct currents. No explanation of this can be offered.

It may be improper to neglect in these experiments the dependence of the threshold on the voltage. This condition may be put in as follows: the maximum of p occurs when $t = \frac{1}{\omega} \tan^{-1} \frac{k}{\omega}$. Since

$V = V_o \sin \omega t$, V will be equal to $\pm \frac{V_o k}{\omega \sqrt{k^2 + \omega^2}}$ at this time. Putting the threshold $= h \pm \alpha V$,

$$h \pm \frac{V_o \alpha k}{\omega \sqrt{k^2 + \omega^2}} = \frac{KV_o}{k^2 + \omega^2}$$

or,

$$h = \frac{K\omega \pm \alpha k}{\omega} \times \frac{V_o}{\sqrt{k^2 + \omega^2}}$$

If K is large compared to $\alpha k/\omega$, as it will probably be for high frequencies, this expression is approximately equal to equation (2)

Evidently in this case as with others it is desirable to make the threshold independent of the voltage if possible in order to attain the greatest simplicity. What conditions existed with the present experiments are not known.

It may be quite improper also to consider that p may take on negative values as has been tacitly assumed in the analysis. In assuming this it must be supposed that the value of p at a given point due to a current in one direction appears to be negative when the current is reversed and p commences to be built up by the current in the new direction. The legitimacy of this assumption cannot safely be decided by the fit of equation (28) with the data. A knowledge of what p actually represents may be necessary, but lacking this a good deal of information may be obtainable experimentally, by finding the relation a stimulus applied immediately after an inadequate stimulus in the opposite direction has to the normal.

Equation (28) does not account for the existence of optimal frequencies, nor for the fact that in going from frequencies about 20 to 200 per second the threshold decreases gradually, then increases. No explanation of these phenomena can be offered on the basis of the present hypothesis. It is not improbable, however, that these effects are primarily due to something quite apart from the postulated mechanism. The electrotonus, for example, will be with alternating current a periodic phenomena as will any other secondary effects depending on the current alone. The existence of periodic phenomena which are known to be mutually related suggests a possibility of an optimal frequency.

On the whole the agreement of the data with equation (28) must be regarded with some distrust as regards its significance. The probability that it is fortuitous is small, but the situation is undoubtedly much more complex than with the unidirectional stimuli, and more should be known about the meaning of k before it can be assumed that it can take on a group of values whose members apply to different frequency ranges. On the other hand, however, if equation (28) is found to be valid quite generally it may be suggestive in determining the nature of the physical mechanism.

The Transient Term

In Experiment *b* of Table II are given the voltages required for the short tetani which always appear with high frequency currents before the threshold for steady tetanus is reached. It will be observed that these values diverge more and more from the steady tetanus values as the frequency increases. This is to be expected from the nature of the transient term of equation (27). No attempt has been made to get a numerical check of this transient phenomenon because of algebraic difficulties. The prediction of equation (27) that the twitch threshold will be farther and farther below the tetanus threshold, is qualitatively fulfilled by the data.

General Considerations

The methods employed here may be considered to be highly artificial, but quite the contrary is true. In attacking the problem there are but three possibilities presented in regard to the action of the current. Its action depends on its magnitude or on its energy or on both. This allows of three possible initial hypotheses:

$$\frac{dp}{dt} = KV, \text{ or } \frac{dp}{dt} = KV^2, \text{ or } \frac{dp}{dt} = f(V, V^2)$$

The third would be tried only in event of the failure of the other two, as such a relation would be unusual. The *second* has been used; e.g., by Lapicque, and by Lassalle (Evans, 1930) and de Almeida (1931). The nature of the problem indicates, however, the use of the first, since the movement of ions is a function of the current or voltage rather than of the energy. A serious objection, also, to using the energy is that the excitation process depends on the direction of the current. This fact cannot safely be ignored.

The nature of the second hypothesis is imposed by observation. It is a fact that the local excitatory process decays spontaneously. The view may be taken that it decays only when it is not being built up (i.e., after the current has been stopped), or only after it has reached a certain magnitude. The former view is definitely at variance with the facts, because the local excitatory process is not directly cumulative during stimulation. The latter view is of the nature of a very special hypothesis. *A priori*, if the local excitatory process decays at all and

decays to completion, there is no reason why it should not decay whenever it exists and no reason why it should not decay according to the same law at all times. This being admitted there remains only to discover the law of decay. The one chosen, $dp/dt = -kp$, which appears to be entirely adequate, is the simplest one of the class of relations $-dp/dt = \text{function of } p$. Had this not been adequate the procedure would have been to try more complicated functions of p .

The third hypothesis, that the local excitatory process must attain a certain liminal value, in order that the stimulus be adequate, is the only one suggested by the phenomenon. It can be avoided only by postulating that the response process to any given stimulus is different from those for all other stimuli.

It has already been pointed out that the direct current formula used here is closely related to those of Lapicque (1907) and Hoorweg, and to Weiss's law, and is exactly equivalent to Hill's equation (1910). The relating of formulae which fit or nearly fit the same data depends only on mathematical dexterity in reducing them to equivalent forms. It follows, however, that much of the present analysis must be closely related to older work, particularly that based on the leaky condenser idea which is just a particular case of the present hypotheses. No attempt has been made to discover or to point out all the possible parallelisms. The only novelty that is claimed for the present analysis is, that it is more complete than any other which in any of its aspects is mathematically equivalent, and that it is based on a more simple and more general point of view. It is thought that the hypotheses used here are the simplest that have any promise of being adequate. That they appear to be entirely adequate in so many cases indicates strongly that they embrace the main features of the excitatory process. It is unlikely that any particular case will involve anything more for its elucidation than minor additions to the general hypotheses.

SUMMARY

Hypotheses previously used (Blair, 1932) in deriving formulae for stimulation by direct currents are applied to other forms of electrical stimuli. This consists in considering solutions of the equation

$$\frac{dp}{dt} = KV - kp$$

where p is assumed to represent the local excitatory process, V is the voltage of the stimulus and K and k are constants. The solutions are discussed in regard to condenser discharges, linearly rising currents, exponentially rising currents, and alternating currents. New experimental work with alternating currents of frequencies above 400 per second on the sciatic gastrocnemius of the frog is related to the formulae.

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INDEX TO AUTHORS

- ABRAMSON, HAROLD A.** Electrokinetic phenomena. V. A small but constant source of error in measurements of viscosity, 279
 —. VI. Relationship between electric mobility, charge, and titration of proteins, 575
 — and GROSSMAN, E. B. Electrokinetic phenomena. VII. Relationship between electric mobility, charge, titration curve, and optical rotation of protein, 605
- ALLISON, JAMES B.** Stimulation by hydrochloric acid and by the normal aliphatic acids in the sunfish *Eupomotis*, 621
 —. See COLE and ALLISON, 119
- ANSON, M. L., and MIRSKY, A. E.** The effect of denaturation on the viscosity of protein systems, 341
- ARNOLD, WILLIAM.** See EMERSON and ARNOLD, 391
- BARMORE, MARK, and LUCK, JAMES MURRAY.** The rôle of phosphate in biological oxidations, 97
- BLAIR, H. A.** On the intensity-time relations for stimulation by electric currents. I, 709
 —. On the intensity-time relations for stimulation by electric currents. II, 731
- BOYKIN, JAMES T.** See SHOUP and BOYKIN, 107
- BURK, DEAN.** See LINEWEAVER, BURK, and HORNER, 497
- CASTLE, E. S.** On "reversal" of phototropism in *Phycomyces*, 487
- COLE, KENNETH S.** Electric phase angle of cell membranes, 641
- COLE, WILLIAM H.** Stimulation by the salts of the normal aliphatic acids in the rock barnacle *Balanus balanoides*, 611
 — and ALLISON, JAMES B. Stimulation by hydrochloric acid in the catfish, *Schilbeodes*, 119
- COULTER, CALVIN B.** See STONE and COULTER, 629
- CROZIER, W. J., and PINCUS, G.** Analysis of the geotropic orientation of young rats. III. Part 1, 201
 — and —. Analysis of the geotropic orientation of young rats. III. Part 2, 225
 — and —. Analysis of the geotropic orientation of young rats. IV, 243
 — and —. Analysis of the geotropic orientation of young rats. V, 421
 — and —. Analysis of the geotropic orientation of young rats. VI, 437
 —. See HOAGLAND and CROZIER, 15
- DAMON, E. B.** Dissimilarity of inner and outer protoplasmic surfaces in *Valonia*. III, 525

- EMERSON, ROBERT, and ARNOLD, WILLIAM. A separation of the reactions in photosynthesis by means of intermittent light, 391
- FERGUSON, A. L., and SCHLUCHTER, A. W. The combining weight of gelatin as an acid, 463
— and —. The mobility of the gelatinate ion, 477
- GOWEN, JOHN W., and TOBEY, ELMER R. On the mechanism of milk secretion. The influence of insulin and phlorhizin, 67
— and —. Studies on milk secretion. The influence of inanition, 45
- GREENBERG, DAVID M., and MACKEY, M. A. The sol-gel transformation in gelatin, 161
- GROSSMAN, E. B. See ABRAMSON and GROSSMAN, 605
- GRUNDFEST, HARRY. The sensibility of the sunfish, *Lepomis*, to monochromatic radiation of low intensities, 307
—. The spectral sensibility of the sunfish as evidence for a double visual system, 507
- HARVEY, E. NEWTON. Photocell analysis of the light of the Cuban elaterid beetle, *Pyrophorus*, 139
— and LOOMIS, ALFRED L. High speed photomicrography of living cells subjected to supersonic vibrations, 147
- HECHT, SELIG, and WOLF, ERNST. Intermittent stimulation by light. I. The validity of Talbot's law for *Mya*, 369
- HITCHCOCK, DAVID I. The combination of a standard gelatin preparation with hydrochloric acid and with sodium hydroxide, 125
- HOAGLAND, H., and CROZIER, W. J. Geotropic excitation in *Helix*, 15
- HOLLENBERG, G. J. Some physical and chemical properties of the cell sap of *Halicystis ovalis* (Lyngbye) Areschoug, 651
- HORNER, C. KENNETH. See LINEWEAVER, BURK, and HORNER, 497
- HUSSEY, RAYMOND. See THOMPSON and HUSSEY, 9
—. See THOMPSON, JOHNSON, and HUSSEY, 1
- JACQUES, A. G., and OSTERHOUT, W. J. V. The accumulation of electrolytes. IV. Internal versus external concentrations of potassium, 537
- JOHNSON, CARL E. See THOMPSON, JOHNSON, and HUSSEY, 1
- KLEINER, ISRAEL S. See TAUBER and KLEINER, 155
- KRUEGER, ALBERT P. The heat inactivation of antistaphylococcus bacteriophage, 363
—. See NORTHROP and KRUEGER, 329
- LINEWEAVER, HANS, BURK, DEAN, and HORNER, C. KENNETH. The temperature characteristic of respiration of *Azotobacter*, 497
- LOOMIS, ALFRED L. See HARVEY and LOOMIS, 147
- LUCK, JAMES MURRAY. See BARMORE and LUCK, 97
- LUYET, BASILE J. Variation of the electric resistance of plant tissues for alternating currents of different frequencies during death, 283
- MACKEY, M. A. See GREENBERG and MACKEY, 161

AUTHORS

- MARTIN, DONALD S. The oxygen consumption of *Escherichia coli* during the lag and logarithmic phases of growth, 691
- MIRSKY, A. E. See ANSON and MIRSKY, 341
- NELSON, J. M., PALMER, ELIZABETH T., and WILKES, B. G. Similarity of the kinetics of invertase action *in vivo* and *in vitro*, 491
- NORTHROP, JOHN H. The presence of a gelatin-liquefying enzyme in crude pepsin preparations, 29
- and KRUEGER, ALBERT P. The rôle of intracellular bacteriophage in lysis of susceptible staphylococci, 329
- OSTERHOUT, W. J. V., and STANLEY, W. M. The accumulation of electrolytes. V. Models showing accumulation and a steady state, 667
- See JACQUES and OSTERHOUT, 537
- PALMER, ALBERT H. The adsorption of gelatin by collodion membranes, 551
- PALMER, ELIZABETH T. See NELSON, PALMER, and WILKES, 491
- PINCUS, G. See CROZIER and PINCUS, 201, 225, 243, 421, 437
- RAHN, OTTO. A chemical explanation of the variability of the growth rate, 257
- RASHEVSKY, N. On the physical nature of "cytotropism" and allied phenomena and their bearing on the physics of organic form, 289
- SCHLUCHTER, A. W. See FERGUSON and SCHLUCHTER, 463, 477
- SHOUP, CHARLES S., and BOJAMES T. The insensitivity of *Paramecium* to cyanide and of iron on respiration, 4
- STANLEY, W. M. See OSTERHOUT and STANLEY, 341
- STONE, FLORENCE M., and COUCALVIN B. Porphyrine compounds derived from bacteria, 4
- TANG, PEI-SUNG. The effect of CO and light on the oxygen consumption and on the product CO₂ by germinating seeds of *Lupinus albus*, 4
- On the respiratory quotient of *Lupinus albus* as a function of temperature, 4
- A respirometer vessel for the study of metabolism of seeds, 4
- Temperature characteristics of the production of CO₂ by germinating seeds of *Lupinus albus* and *Zea mays*, 4
- TAUBER, HENRY, and KLEISRAEL S. Studies on crystalline urease. IV. The "antitrypsin" property of crystalline urease, 4
- THOMPSON, WILLIAM R., and HURAYMOND. The effect of ultraviolet radiation on amylase in solution, 4
- JOHNSON, CARL E., and HURAYMOND. A viscosimetric method of estimating enzyme concentration with special reference to amylase, 4
- TOBEY, ELMER R. See GOWENLOCK and TOBEY, 4
- UPTON, MORGAN. The effect of added loads upon the geotropic orientation of young guinea

- | | |
|---|---|
| <p>WHITAKER, D. M. On the
rate of oxygen consumption by
fertilized and unfertilized eggs. I.
<i>Fucus vesiculosus</i>, 167</p> <p>— II. <i>Cumingia tellinoides</i>, 183</p> <p>— III. <i>Nereis limbata</i>, 191</p> | <p>WILKES, B. G. See NELSON, PAL-
MER, and WILKES, 491</p> <p>WOLF, ERNST. See HECHT and
WOLF, 369</p> <p>WYCKOFF, RALPH W. G. The kill-
ing of colon bacilli by ultraviolet
light, 351</p> |
|---|---|

INDEX TO SUBJECTS

- ACID**, gelatin, combining weight, 463
- Aliphatic acids, normal, salts, stimulating rock barnacle, *Balanus balanoides*, 611
- — —, stimulation in sunfish, *Eupomotis*, 621
- Amylase concentration estimated by viscosimetric method, 1
- in solution, effect of ultraviolet radiation, 9
- Antistaphylococcus bacteriophage, heat inactivation, 363
- Antitryptic property of crystalline urease, 155
- Azotobacter*, respiration, temperature characteristic, 497
- BACILLUS coli**, killing by ultraviolet light, 351
- Bacteria, porphyrine compounds derived from, 629
- Bacteriophage, antistaphylococcus, heat inactivation, 363
- , intracellular, rôle in lysis of susceptible staphylococci, 329
- Balanus balanoides*, stimulation by salts of normal aliphatic acids, 611
- Barnacle, rock, *Balanus balanoides*, stimulation by salts of normal aliphatic acids, 611
- Beetle, Cuban elaterid, *Pyrophorus*, photocell analysis of light, 139
- Biological oxidations, rôle of phosphate, 97
- CARBON** dioxide production by germinating seeds of *Lupinus albus*, effect of carbon monoxide, 655
- Carbon dioxide production by germinating seeds of *Lupinus albus*, effect of light, 655
- — — — germinating seeds of *Lupinus albus*, temperature characteristics, 87
- — — — germinating seeds of *Zea mays*, temperature characteristics, 87
- monoxide, effect on oxygen consumption and production of carbon dioxide by germinating seeds of *Lupinus albus*, 655
- Catfish, *Schilbeodes*, hydrochloric acid stimulation, 119
- Cell membranes, electric phase angle, 641
- sap of *Halicystis ovalis* (Lyngbye) Areschoug, chemical properties, 651
- — — *Halicystis ovalis* (Lyngbye) Areschoug, physical properties, 651
- Cells, living, subjected to supersonic vibrations, high speed micrography, 147
- Charge, electric, and electric mobility, titration curve, and optical rotation of protein, relationship, 605
- , —, — — mobility, and titration of proteins, relationship, 575
- Chemical explanation of variability of growth rate, 257
- properties of cell sap of *Halicystis ovalis* (Lyngbye) Areschoug, 651
- Collodion membranes, adsorption of gelatin, 551
- Colon bacillus. *See Bacillus coli*.
- Crystalline urease, 155
- —, antitryptic property, 155

- Cuban elaterid beetle, *Pyrophorus*,
 photocell analysis of light, 139
- Cumingia tellinoides*, rate of oxygen
 consumption by fertilized eggs,
 183
- — — oxygen consumption by
 unfertilized eggs, 183
- Cyanide, insensitivity of *Paramecium*
 to, 107
- Cytotropism and allied phenomena,
 physical nature and bearing on
 physics of organic form, 289
- D**EATH, variation of electric resis-
 tance of plant tissues for alternat-
 ing currents of different frequencies
 during, 283
- Denaturation, effect on viscosity of
 protein systems, 341
- E**GGs, fertilized, of *Cumingia telli-*
noides, rate of oxygen consump-
 tion, 183
- , —, — *Fucus vesiculosus*, rate of
 oxygen consumption, 167
- , —, — *Nereis limbata*, rate of oxy-
 gen consumption, 191
- , unfertilized, of *Cumingia tellinoides*,
 rate of oxygen consumption,
 183
- , —, — *Fucus vesiculosus*, rate of
 oxygen consumption, 167
- , —, — *Nereis limbata*, rate of oxy-
 gen consumption, 191
- Elaterid beetle, Cuban, *Pyrophorus*,
 photocell analysis of light, 139
- Electric charge, electric mobility, ti-
 tration curve, and optical rotation
 of protein, relationship, 605
- —, mobility, and titration of pro-
 teins, relationship, 575
- mobility, charge, titration curve,
 and optical rotation of protein, rela-
 tionship, 605
- —, —, and titration of proteins, re-
 lationship, 575
- phase angle of cell membranes,
 641
- Electric resistance of plant tissues,
 variation for alternating currents
 of different frequencies during
 death, 283
- Electricity, intensity-time relations
 for stimulation by electric currents,
 709, 731
- Electrokinetic phenomena,
 279, 575, 605
- Electrolytes, accumulation,
 537, 667
- , models showing accumulation and
 steady state, 667
- Enzyme concentration, viscosimetric
 method of estimating, with special
 reference to amylase, 1
- , gelatin-liquefying, presence in
 crude pepsin preparations, 29
- Enzymes, effect of radiations from
 mercury arc in quartz, 9
- Escherichia coli*, oxygen consumption
 during lag phase of growth,
 691
- —, — — during logarithmic phase
 of growth, 691
- Eupomotis*, stimulation by hydro-
 chloric acid, 621
- , — — normal aliphatic acids,
 621
- F**ERTILIZED eggs of *Cumingia*
tellinoides, rate of oxygen consump-
 tion, 183
- — — *Fucus vesiculosus*, rate of
 oxygen consumption, 167
- — — *Nereis limbata*, rate of oxygen
 consumption, 191
- Fucus vesiculosus*, rate of oxygen con-
 sumption by fertilized eggs,
 167
- —, — — oxygen consumption by
 unfertilized eggs, 167
- G**EL, sol-, transformation in gelatin,
 161
- Gelatin, adsorption by collodion mem-
 branes, 551
- as an acid, combining weight,
 463

- Gelatin preparation, standard, combination with hydrochloric acid, 125
 — — — — — with sodium hydroxide, 125
 —, sol-gel transformation, 161
 Gelatin-liquefying enzyme, presence in crude pepsin preparations, 29
 Gelatinate ion, mobility, 477
 Geotropism in *Helix*, 15
 —, young guinea pigs, effect of added loads, 333
 —, — rats, 201, 225, 243, 421, 437
 Germinating seeds of *Lupinus albus*, effect of carbon monoxide on oxygen consumption and production of carbon dioxide, 655
 — — — *Lupinus albus*, effect of light on oxygen consumption and production of carbon dioxide, 655
 — — — *Lupinus albus*, temperature characteristics for carbon dioxide production, 87
 — — — *Zea mays*, temperature characteristics for carbon dioxide production, 87
 Growth of *Escherichia coli*, lag phase, oxygen consumption during, 691
 — — — — —, logarithmic phase, oxygen consumption during, 691
 — rate, variability, chemical explanation, 257
HALICYSTIS *ovalis* (Lyngbye)
 Areschoug, chemical properties of cell sap, 651
 — — — — —, physical properties of cell sap, 651
 Heat inactivation of antistaphylococcus bacteriophage, 363
Helix, geotropic excitation, 15
 Hydrochloric acid, combination with standard gelatin preparation, 125
 — — stimulation in catfish, *Schilbeodes*, 119
 — — — — — sunfish, *Eupomotis*, 621

- INANITION**, influence on milk secretion, 45
 Insulin, influence on mechanism of milk secretion, 67
 Intracellular bacteriophage, rôle in lysis of susceptible staphylococci, 329
 Invertase action, similarity of kinetics *in vivo* and *in vitro*, 491
In vitro and *in vivo*, similarity of kinetics of invertase action, 491
 — *vivo* and *in vitro*, similarity of kinetics of invertase action, 491
 Ion, gelatinate, mobility, 477
 Iron, effects on respiration of *Paramecium*, 107
KINETICS of invertase action, similarity *in vivo* and *in vitro*, 491
LEPOMIS, sensibility to monochromatic radiation of low intensities, 307
 Light, effect on oxygen consumption and carbon dioxide production by germinating seeds of *Lupinus albus*, 655
 —, intermittent, for separation of reactions in photosynthesis, 391
 — of Cuban elaterid beetle, *Pyrophorus*, photocell analysis, 139
 —, sensibility of sunfish, *Lepomis*, to monochromatic radiation of low intensities, 307
 —, stimulation, intermittent, 369
 —, ultraviolet, effect on amylase in solution, 9
 —, —, killing of colon bacilli, 351
Lupinus albus, germinating seeds, effect of carbon monoxide on oxygen consumption and production of carbon dioxide, 655
 — — — — —, effect of light on oxygen consumption and production of carbon dioxide, 655
 — — — — —, temperature characteristics for carbon dioxide production, 87

- Lupinus albus*, respiratory quotient as function of temperature, 561
 Lysis, susceptible staphylococci, rôle of intracellular bacteriophage, 329

- M**EMBRANES, cell, electric phase angle, 641
 —, collodion, adsorption of gelatin, 551
 Mercury arc in quartz, effect of radiations on enzymes, 9
 Metabolism of seeds, respirometer vessel for study, 571
 Milk secretion, 45
 — —, influence of inanition, 45
 — —, — — insulin, 67
 — —, — — phlorhizin, 67
 — —, mechanism, 67
Mya, validity of Talbot's law, 369

- N**EREIS *limbata*, rate of oxygen consumption by fertilized eggs, 191
 — —, — — oxygen consumption by unfertilized eggs, 191

- O**PTICAL rotation of protein, and electric mobility, charge, and titration curve, relationship, 605

- Orientation, geotropic, of young guinea pigs, effect of added loads, 333
 —, —, — young rats, 201, 225, 243, 421, 437

- Oxidations, biological, rôle of phosphate, 97

- Oxygen consumption by fertilized eggs of *Cumingia tellinoides*, rate, 183
 — — — — eggs of *Fucus vesiculosus*, rate, 167
 — — — — eggs of *Nereis limbata*, rate, 191
 — — — unfertilized eggs of *Cumingia tellinoides*, rate, 183

- Oxygen consumption by unfertilized eggs of *Fucus vesiculosus*, rate, 167
 — — — — eggs of *Nereis limbata*, rate, 191
 — — of *Escherichia coli* during lag phase of growth, 691
 — — — *Escherichia coli* during logarithmic phase of growth, 691
 — — — germinating seeds of *Lupinus albus*, effect of carbon monoxide, 655
 — — — — seeds of *Lupinus albus*, effect of light, 655

- P**ARAMECIUM, effects of iron on respiration, 107
 —, insensitivity to cyanide, 107
 Pepsin preparations, crude, presence of gelatin-liquefying enzyme, 29
 Phlorhizin, influence on mechanism of milk secretion, 67
 Phosphate, rôle in biological oxidations, 97
 Photocell analysis of light of Cuban elaterid beetle, *Pyrophorus*, 139
 Photomicrography, high speed, of living cells subjected to supersonic vibrations, 147
 Photosynthesis, reactions, separation by intermittent light, 391
 Phototropism, *Phycomyces*, reversal, 487
Phycomyces, reversal of phototropism, 487
 Physical nature of cytotropism and allied phenomena and bearing on physics of organic form, 289
 — properties of cell sap of *Halicystis ovalis* (Lyngbye) Areschoug, 651
 Physics of organic form, bearing of cytotropism and allied phenomena, 289
 Plant tissues, variation of electric resistance for alternating currents of different frequencies during death, 283

SUBJECTS

- Porphyrine compounds derived from 629
 bacteria, internal *versus* external 537
 Potassium, concentrations, 605
 Protein, optical rotation, and electric mobility, charge, and titration 341
 — systems, viscosity, effect of denaturation, electric mobility, charge, and titration, relationship, 575
 Protoplasm, inner and outer surfaces, dissimilarity in *Valonia*, 525
Pyrophorus, Cuban elaterid beetle, photocell analysis of light, 139
- QUARTZ**, mercury arc in, effect of radiations on enzymes, 9
- RADIATION** of low intensities, monochromatic, sensibility of sunfish, *Lepomis*, 307
 —, ultraviolet, effect on amylase in solution, 9
 Radiations from mercury arc in quartz, effect on enzymes, 9
 Respiration, *Azotobacter*, temperature characteristic, 497
 —, *Paramecium*, effects of iron, 107
 Respiratory quotient of *Lupinus albus* as function of temperature, 561
 Respirometer vessel for study of metabolism of seeds, 571
- SALTS** of normal aliphatic acids stimulating rock barnacle, *Balanus balanoides*, 611
 Sap, cell, of *Halicystis ovalis* (Lyngbye) Areschoug, chemical properties, 651
 —, —, — *Halicystis ovalis* (Lyngbye) Areschoug, physical properties, 651
Schilbeodes, hydrochloric acid stimulation, 119
- Secretion, milk, —, —, mechanical 87
 Seeds, germination, temperature characteristics for carbon dioxide production, 87
 —, —, *Zea mays*, temperature characteristics for carbon dioxide production, 571
 —, metabolism, respirometer vessel for study, 571
 — of *Lupinus albus*, germinating, defect of carbon monoxide, oxygen consumption and production of carbon dioxide, 655
 — — —, germinating, effect of light on oxygen consumption, and production of carbon dioxide, 655
 Sodium hydroxide, combination with standard gelatin preparation, 25
 Sol-gel transformation in gelatin, 25
 Spectral sensibility of sunfish, dependence for double visual system, 1
- Staphylococci, susceptible, lysis of intracellular bacteriophage
- Stimulation, electric current, time relations, 705
 —, hydrochloric acid, in calcium *Schilbeodes*, —, —, — sunfish, *Eupomotis*, —, intermittent, by light, —, normal aliphatic acid, in sunfish, *Eupomotis*, —, salts of normal aliphatic acids, rock barnacle, *Balanus balanoides*, 6: 62
 Sunfish, *Eupomotis*, hydrochloric acid stimulation, 621
 —, —, normal aliphatic acid stimulation, 621
 —, *Lepomis*, sensibility to monochromatic radiation of low intensities, 307

